Binding properties of the cell wall of Saccharomyces cerevisiae.

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BINDING PROPERTIES OF THE CELL WALL OF

SACCHAROMYCES CEREVISIAE

submitted by

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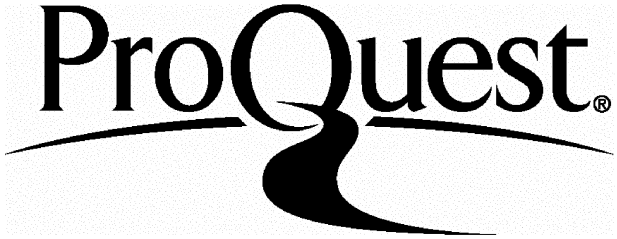

Dedicated to my Father

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SUMMARY

PART I

A study was made of binding of an antifoam preparation containing polydimethylsiloxane (PDS) to cells of *Saccharomyces cerevisiae* NCYC 366. These cells bound approximately 2.1 µg PDS per 2×10^7 organisms when suspended in 0.1 M KH_2PO_4 buffer (pH 4.5) containing Antifoam M-10 (to give 19 µg PDS per ml). A decrease in the concentration of any one of the two emulsifiers, polyoxyethylene sorbitan monostearate or glycerol monostearate caused an increase in the saturation concentration of PDS bound by the organisms, while a decrease in the concentration of the thickener, sodium carboxymethyl cellulose caused a decrease. The sites involved in binding PDS were indicated to be located in the cell-wall phosphomannan-protein of the organisms by studies made on PDS binding by whole cells, PDS release by saturated organisms, analyses of isolated walls and surface properties of organisms, before and after chemical and enzymic treatments. The surface charge of the organisms at different pH values had no effect on PDS binding. Binding of PDS had a masking effect on the electrophoretic mobility, binding of antibody and binding of concanavalin A by the organisms, but had no effect on the release of invertase.

PART II

An examination was made of the ability of each of 4 strains of *Saccharomyces cerevisiae* to flocculate following the excision of the phosphodiester linkages of cell-wall phosphomannan. Treatment of isolated walls of each of the 4 strains with hydrofluoric acid (58 - 62%, v/v) removed most of the phosphorus without extensive losses of other components. Treatment of whole cells with the same reagent increased the sedimentation rates of both flocculent and non-flocculent cells. The presence of Ca^{2+} ions was found to be essential for the expression of flocculence either inherent or induced by hydrofluoric acid treatment. Esterification of surface carboxyl groups decreased the sedimentation rates of both untreated flocculent cells and hydrofluoric acid-treated organisms. Inclusion of mannose in the suspending medium deflocculated untreated flocculent cells but failed to deflocculate hydrofluoric acid-treated organisms. The phosphorus contents of the outer layers of the cell-wall as indicated by the electrophoretic mobility at pH 4.0 or the amount of calcium bound by isolated walls was not related to the flocculation characteristics of the organisms.

INTRODUCTION

COMPOSITION AND STRUCTURE OF THE YEAST CELL WALL

Of the numerous topics in yeast biochemistry, composition and structure of the yeast cell wall has found particular interest among microbial biochemists. Current knowledge of the nature of the yeast cell wall is based largely on the combined evidence obtained from chemical and physical studies, enzymic degradations of the cell wall and from cytological investigations. Much of this work has been done on strains of *Saccharomyces cerevisiae* (baker's and brewer's yeasts) due to their ready availability. Studies on other yeasts have shown some diversity in structure and composition (Phaff, 1971).

Early investigators generally subjected whole yeast cells to drastic chemical treatments in order to obtain cell-wall residues or extracts of cell-wall components. With the development of various instruments that effect cell breakage (Wood, 1966; Hughes, Wimpenny & Lloyd, 1971), much progress has been made on the isolation of cell walls. However, isolation of yeast cell walls is a time-consuming operation. To isolate individual components of the cell wall early workers treated whole yeast with hot alkali, which removes the mannan from the wall with some contaminating glycogen, leaving behind a residue composed mainly of glucan. The mannan was precipitated from the extract with cold Fehling's solution and the free polysaccharide liberated by treating this precipitate with dilute alkali. More recently other milder methods have been employed to extract mannan. These methods include autoclaving cells in water or buffer solution (Peat, Whelan & Edwards, 1961a; Peat, Turvey & Doyle, 1961b; Kocourek & Ballou, 1969) and extraction of cells or walls with ethylenediamine (Kessler & Nickerson, 1959; Korn & Northcote, 1960; Klaushofer, Mahr & Szilvinyi, 1961).

A number of yeast cell-wall components have been isolated and studied in detail. Some progress has also been made on the elucidation of types of linkages which hold the major components of the wall together in the intact cell. Reviews on the cell wall of yeast and related aspects have been published by Clarke & Stone (1963), Nickerson (1963), Northcote (1963) Phaff (1963), Bull & Chesters (1966), Bartniki-Garcia (1968), Gorin & Spencer (1968) MacWilliam (1970) and Phaff (1971).

The yeast cell wall is between 100 and 200 nm in thickness (Lyons & Hough, 1970C) and isolated walls of baker's yeast account for about 30% of the dry cell mass (Falcone & Nickerson, 1956). However, two earlier reports (Northcote & Horne, 1952 and Roelofsen, 1953) claimed that samples of baker's yeast contained 15% and 20% of wall, respectively. Brewer's strains of *Saccharomyces cerevisiae* are reported to have wall contents varying between 6% and 27% (Griffin & MacWilliam, 1969).

The native cell wall represents a very complex, heterogeneous organelle and the ultimate building stones of this are glucan, mannan, N-acetyl-D-glucosamine (all or some of it as chitin), protein and lipid. Mill (1966) stated that about 90% of the dry weight of the *Saccharomyces cerevisiae* cell wall is polysaccharide, glucan and mannan being present in about equal amounts. However, it has been established that the ratio of glucan to mannan varies according to growth conditions (Dunwell, Ahmad & Rose, 1961; McMurrough & Rose, 1967) and with the age of the yeast cell. The ratio also varies with the yeast strain (Griffin & MacWilliam, 1969). The rest of the wall is composed of protein (about 8%), lipid (0.8-13%) and N-acetyl-D-glucosamine (0.8 - 0.9%).

Glucan

Glucan, referred to as "yeast cellulose" or "yeast polyose" in early literature, is a polymer of D-glucose, which constitutes the compotent responsible for the shape and rigidity of the yeast cell (Phaff 1971) and was first prepared by Salkowski (1894a). Zechmeister and Toth (1934, 1936) were the first to report the presence of (1-3) linkages in glucan, by methylation analysis. Hassid, Joslyn and McCready (1941) confirmed the presence of (1-3) linkages in baker's yeast glucan, and suggested that the glucan molecule is probably of the closed chain type. They also suggested a predominance of β-linkages in glucan and a molecular weight of 6,500 daltons with a degree of polymerization of 40. The presence of β(1-3) linkages was later confirmed by enzymic studies (Barry & Dillon, 1943).

Bell & Northcote (1950), who studied glucan from a strain of *Saccharomyces cerevisiae*, were the first to obtain evidence on the basis of methylation analysis for a highly branched polysaccharide of large molecular weight. Their evidence indicated unit chains with an average of nine glucose units linked by β(1-3) bonds and with interchain links of the (1-2) type.

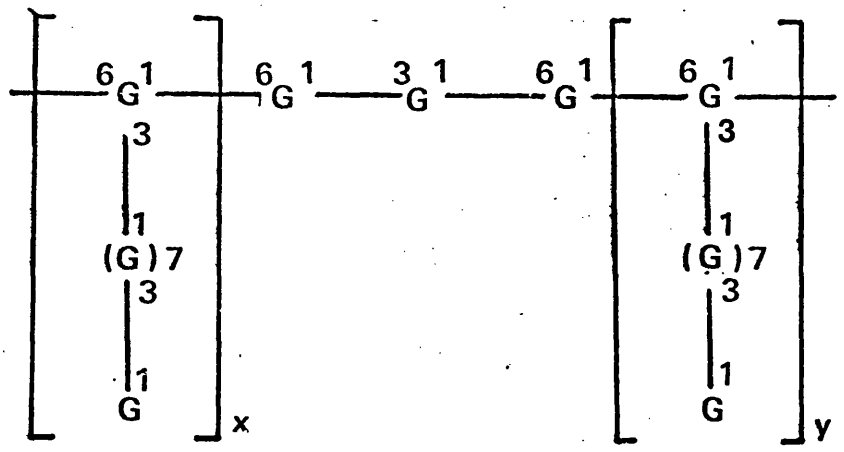
The presence of the β(1-6) type of linkage in glucan was first demonstrated by Peat, Whelan & Edwards (1958a). They subjected baker's yeast glucan to partial hydrolysis with 90% formic acid, and observed oligosaccharides of the gentiobiose series among the hyrolysis products, indicating the presence of β(1-6) type of linkages in the glucan. However they could not confirm the presence of (1-2) linkages, first suggested by Bell & Northcote (1950), due to the absence of any 2-O-β-D-glucopyranosyl-D-glucose

among the hydrolysis products. They envisaged glucan as a linear molecule in which (1-3) and (1-6) linkages occur at random or in sequences such that a group of at least three (1-6) linkages are flanked on either sides by (1-3) bonds. From periodate oxidation studies (Peat *et al.*, 1958a) and later from studies involving toluene-p-sulphonation followed by replacement of the primary tosyl groups by iodine (Peat, Turvey & Evans, 1958b), the primary hydroxyl groups of the glucose units involved in (1-6) linkages were estimated to be 10-20%.

Tanaka (1963) showed by enzymic studies that the ratio of β(1-6) linkages to β(1-3) linkages in baker's yeast glucan was 1:2. However Buecher (1968), who repeated Tanaka's (1963) analysis of baker's yeast cell walls, found that the ratio of linkages susceptible to β(1-6) and β(1-3) glucanases was 1:1.

Analyses of baker's yeast, using periodate oxidation and methylation methods by Misaki & Smith (1963) and Misaki *et al.* (1968), again suggested that the glucan is highly branched. Their studies revealed that the repeating unit of glucan consist of one terminal non-reducing residue, seven β(1-3)-linked non-terminal residues and one branching residue, joined through positions O-1, O-3 and O-6. Misaki *et al.* (1968) proposed the structure shown in Figure 1 as a working model for the glucan.

There is however some difficulty in reconciling this structure with the findings of Peat *et al.* (1958a), Tanaka (1963) and Buecher (1968), because this glucan does not possess a significant number of β(1-6)-linked glucose residues without side chains. Confirmation of the presence of a D-glucose, triply-linked at positions O-1, O-3, and O-6, and the absence of any (1-2) type bonds was supplied by



$x + y = 40 - 50$

Figure 1. Schematic representation of baker's yeast glucan, from Misaki *et al.* (1968). G indicates a β-D-glucopyranosyl residue.

Manners & Patterson (1966). However their studies too indicated a greater proportion of unsubstituted β(1-6)-linked glucose residues in the backbone than fits the model proposed by Misaki *et al.* (1968).

Bacon & Farmer (1968) and Bacon *et al.* (1969) attributed these differences to the presence of a water-soluble β(1-6) glucan, which is removed with the glycogen, on treatment with acetic acid and water in the preparation of yeast glucan. Manners & Masson (1969) characterised this soluble glucan of baker's yeast as a linear β(1-6) polymer containing a very small proportion of β(1-3) linkages and having a degree of polymerization of 140 ± 10. Studies carried out on the residual glucan, after removal of the soluble glucan, by Manners & Masson (1969) revealed that the glucan molecule is less highly branched than proposed by Misaki *et al.* (1968) and that the chains

of β(1-3)-linked glucose residues may be much longer. Manners, Masson & Patterson (1973a), working on four samples of baker's yeast glucan, confirmed the heterogeneity of yeast glucan. They reported that yeast glucan prepared by various treatments of *Saccharomyces cerevisiae* cell walls to remove mannan and glycogen was still heterogeneous. The major component (about 85%) is a branched β(1-3) glucan of high molecular weight (about 240,000 daltons) containing 3% of β(1-6) glucosidic interchain linkages. The minor component is a branched β(1-6) glucan. By comparison of their results with those of other workers, they suggested that different glucan preparations may differ in the degree of heterogeneity, and that the major β(1-3) glucan component may vary considerably in the degree of branching.

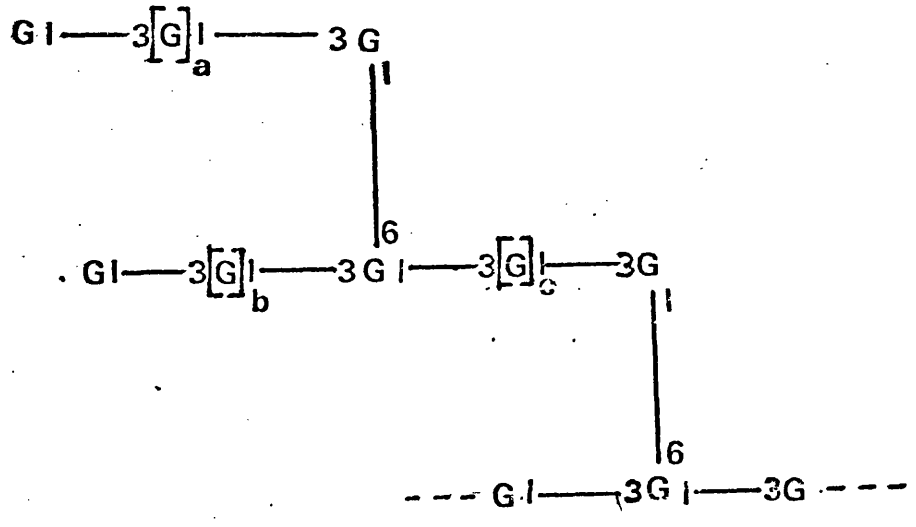


Figure 2 Partial structure of a segment of yeast β(1-3) glucan, from Manners *et al.* (1973a). G indicates a β-D-glucopyranosyl residue. (a+b+c comprises of 60 glucose residues, although the exact lengths of a, b and c are not known.)

Manners *et al.* (1973b) studied the β(1-6) glucan which was said to be the minor, soluble component by Manners *et al.* (1973a). They described this glucan as a polysaccharide having a degree of polymerization of about 130-140 with a highly branched structure and a high proportion of β(1-6) glucosidic linkages. This molecule also contained a smaller proportion of β(1-3) linkages that serve mainly as interchain linkages, but some may also be inter-residue linkages.

Mannan

The outer chain: Yeast mannan, which was termed "yeast gum" in early literature was first prepared by Salkowski (1894b). Haworth, Hirst & Isherwood (1937) and Haworth, Heath & Peat (1941), who extracted mannan by boiling whole yeast with alkali, were the first to study it in detail. They found mannan to be a homogeneous polymer of mannose with a degree of polymerization of 200-400. Methylation analysis revealed mannan to be a highly branched molecule and its high dextrorotation indicated the presence of α-linkages, although the presence of β-linkages could not be excluded. Based on an analysis of the methylated sugars present after hydrolysis, they postulated the three repeating units. These structures show that the side chains could be single mannose residues, or two, or a combination of one and two.

In these studies, the presence of (1-6) linkages in unsubstituted mannose residues was indicated by the finding of a small amount of 2,3,4-trimethylmannose among the hydrolysis products of methylated mannan. This was explained by assuming that the terminal non-reducing end of the chain occurs at position a in Figure 3, which would yield this methylated derivative.

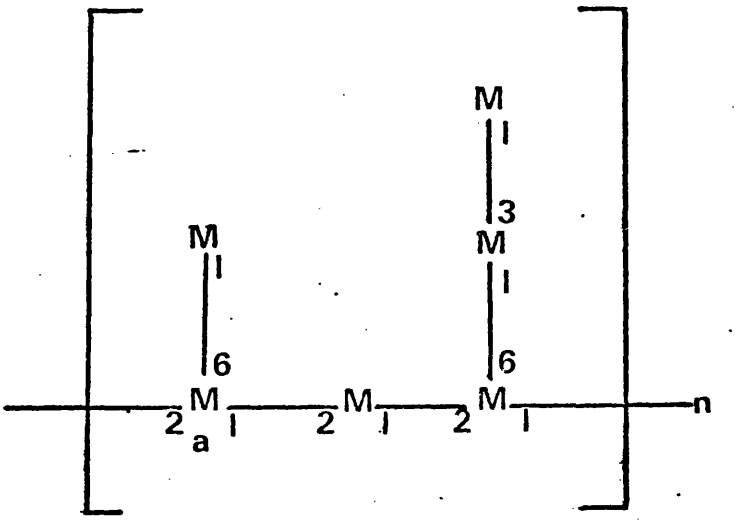


Figure 3 One of the three possible structures for the repeating unit in baker's yeast mannan, from Haworth *et al.* (1937, 1941). M indicates an α-D-mannopyranosyl residue. See text for details.

Lindstedt (1945) and Cifonelli & Smith (1955) confirmed the results of Haworth and his co-workers by periodate oxidation technique and methylation analysis respectively. However, Peat *et al.* (1961a) who studied mannan extracted by autoclaving baker's yeast in citrate buffer at pH 7.0, using a technique involving partial hydrolysis, fractionation and examination of fragments, concluded that the backbone of the highly branched mannan must contain α(1-6) and not α(1-2) linkages as postulated by Haworth *et al.* (1937, 1941). Much later, Jones & Ballou (1968) supplied proof for the existence of α(1-6) linkages in the backbone of the mannan molecule from enzymic studies. Peat *et al.* (1961b), who

studied fragmentation of mannan by acetolysis, provided direct evidence for the presence of α(1-2) as well as α(1-6) bonds in the resulting disaccharides. From these and other data, Peat *et al.* (1961b) put forward the structure in Figure 4, to represent the simplest repeating unit in baker's yeast mannan.

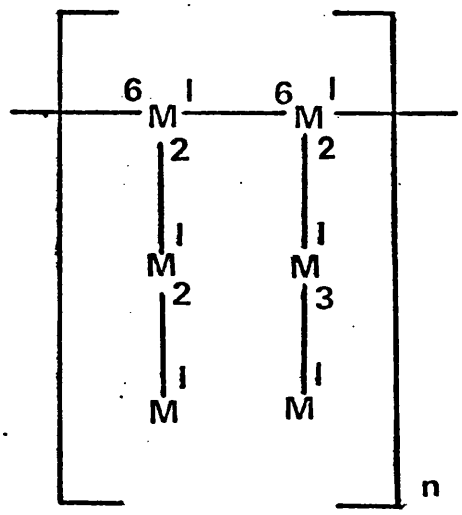


Figure 4 Repeating unit in baker's yeast mannan, from Peat *et al.* (1961b). M indicates an α-D-mannopyranosyl residue.

To explain the finding of 2,3,4-trimethyl-D-mannose in the hydrolysate of methylated mannan they postulated the presence of an occasional mannose residue in the backbone not carrying a side chain. Alternatively, it might constitute the reducing unsubstituted end group of the backbone chain.

Lee & Ballou (1965), who carried out controlled acetolysis of baker's yeast mannan, found in agreement with Gorin & Perlin (1956) that this treatment cleaved α(1-6) linkages of the backbone, yielding oligosaccharides none of which contained (1-6) bonds. The resulting oligosaccharides were identified as mannobiose, mannotriose (both with α(1-2) linkages) and a novel tetrasaccharide, namely O-α-D-mannopyranosyl-(1-3)-O-α-D-mannopyranosyl-(1-2)-O-α-D-mannopyranosyl-(1-2)-D-mannopyranose. Later Stewart, Mendershausen & Ballou (1968) identified another trisaccharide, O-α-D-mannopyranosyl-(1-3)-O-α-D-mannopyranosyl-(1-2)-D-mannopyranose, as an integral part of the mannan molecule. From these results Stewart & Ballou (1968) postulated the basic structure shown in Figure 5 for baker's yeast mannan, without implying any particular ratio or order of the units in the chain.

Chieko, Numokawa & Akiyama (1971), who studied mannan extracted from saké yeast which is classified as *Saccharomyces cerevisiae*, reported an interesting difference from baker's yeast mannan. This was the release of a mannopentaose from this mannan on acetolysis. This newly found side chain residue was suggested to be linked to the main chain by an (1-2) bond with two successive (1-3) bonds at the end group.

Presence of phosphorus in mannan: The presence of phosphorus in mannan isolated from baker's yeast was reported by Northcote & Horne (1952) and later confirmed by Lindquist (1953). Eddy (1958), who studied mannan extracted from walls of *Saccharomyces cerevisiae*, found the phosphorus content in the mannan to be 1% or less. He also suggested that phosphorus in the form of phosphate, forms an integral part of the mannan molecule.

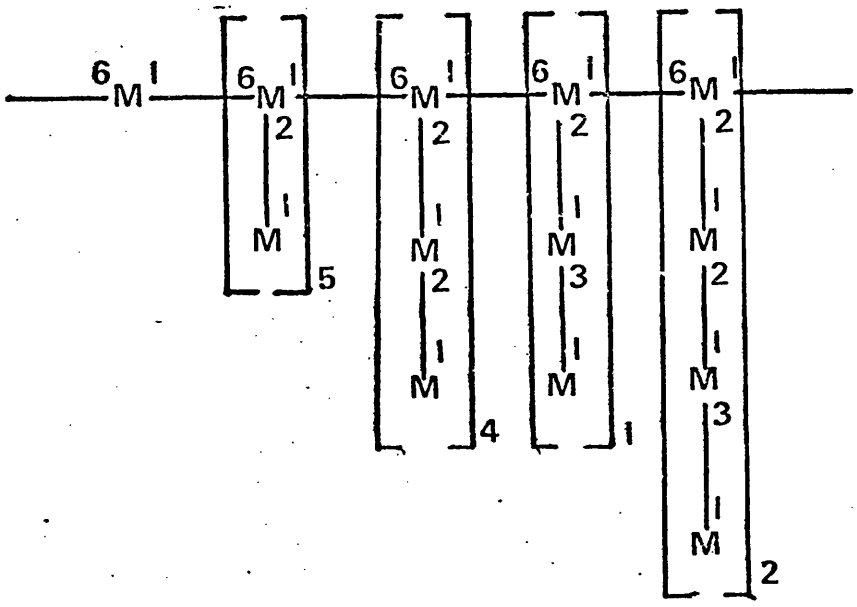


Figure 5. Schematic structure of baker's yeast mannan, from Stewart & Ballou (1968). The subscripts outside the brackets indicate the average molecular proportions of the various types of side chains. M indicates an α-D-mannopyranosyl residue.

Mannose-6-phosphate was identified as a product of acid hydrolysis of mannan from *Saccharomyces cerevisiae* by Mill (1966). This indicated that, at least in baker's yeast, the phosphate appears to be linked to the 6-position of the terminal non-reducing mannose residue of the main chain or to the (1-2) or (1-3) linked residues of the side chain. Mill (1966) also found that the phosphate was di-esterified and that the second (unknown) linkage was very acid-labile, possibly representing a hemi-acetal

bond to C-1 of another mannose residue.

Cawley & Letters (1968) obtained a phosphoglycopeptide from walls of *Saccharomyces cerevisiae*, in which the phosphate was still present as a diester. Their studies suggested that the phosphodiester groups were linked between C-6 of one mannose unit and C-1 of another which in turn was thought to be linked glycosidically at C-2 to another mannose residue. Phosphate esterified at C-6 on a mannose residue in a purified mannan fraction from baker's yeast , was also identified by Sentandreu & Northcote (1968).

The diesterified phosphate in mannan from baker's yeast and another strain of *Saccharomyces cerevisiae* (S288C) was reported to be converted to the monoester form, with the release of an equimolar amount of mannose and mannobiose in variable ratios following mild acid hydrolysis (Thieme & Ballou, 1971; Colonna & Lampen, 1974a). The mannobiose was later found to be α-D-mannopyranosyl-(1-3)-D-mannose (Rosenfeld & Ballou, 1974). These results indicate that one ligand to the phosphodiester group is mannose or mannobiose. Rosenfeld & Ballou (1974), who studied the resulting monoester phosphomannan after the release of mannose and mannobiose showed that this yields a mannotetraose-phosphate on partial acetolysis. By a series of degradation reactions, the phosphate was shown to be attached to the mannose unit next to the one at the reducing end of the fragment. The exact poisition of attachment was established by a selective degradation (Cawley, Harrington & Letters, 1972; Rosenfeld, 1974) to be C-6 of the mannose residue.

Base-Labile oligosaccharides: Sentandreu & Northcote (1968)

reported the release of mannose and a mixture of disaccharides when bulk mannan from *Saccharomyces cerevisiae* was treated with 0.1N NaOH at 20°C - 40°C for 24 hours. This treatment was reported by Thieme & Ballou (1971) and Cawley *et al.* (1972) to release even tri-and tetrasaccharides. Colonna & Lampen (1974b) confirmed that this mild treatment caused bulk mannan from *Saccharomyces cerevisiae* to release mono-, di-, tri- and possibly tetrasaccharides. The exact structure of these base-labile oligosaccharides has been studied (Nakajima & Ballou, 1974a) and reported to be identical with those of the di-, tri- and tetrasaccharide fragments produced by the acetolysis of the mannan main chain.

The inner-core: The discovery of an inner-core structure in yeast mannan has been reported for the mannan obtained from mnn2 mutant of *Saccharomyces cerevisiae* X2180 (Raschke *et al.*, 1973; Nakajima & Ballou, 1974b). Acetolysis and enzymic studies have revealed that the majority of this mannan was an unsubstituted α(1-6)-linked chain. However the rest of the molecule retained a branch structure which was characterized as the inner-core of the main polymannose chain. This portion of the molecule was assumed to yield di-, tri- and tetrasaccharides on acetolysis.

With the discovery of the inner-core, a more detailed description of *Saccharomyces cerevisiae* mannan can now be presented, in which the carbohydrate moiety of the molecule is differentiated into three parts; the outer chain, the inner-core

and the base-labile oligosaccharides.

In a very recent article, Ballou (1976) stated that, characteristically, baker's yeast mannan is composed of long D-mannose chains in α(1-6) linkages, with short side chains in α(1-2) and α(1-3) linkage. These macromolecules are attached to asparagine in protein. In addition, this mannan has short oligomannoside units with α(1-2) and α(1-3) linkages, that are linked to serine and threonine in protein (Fig. 6).

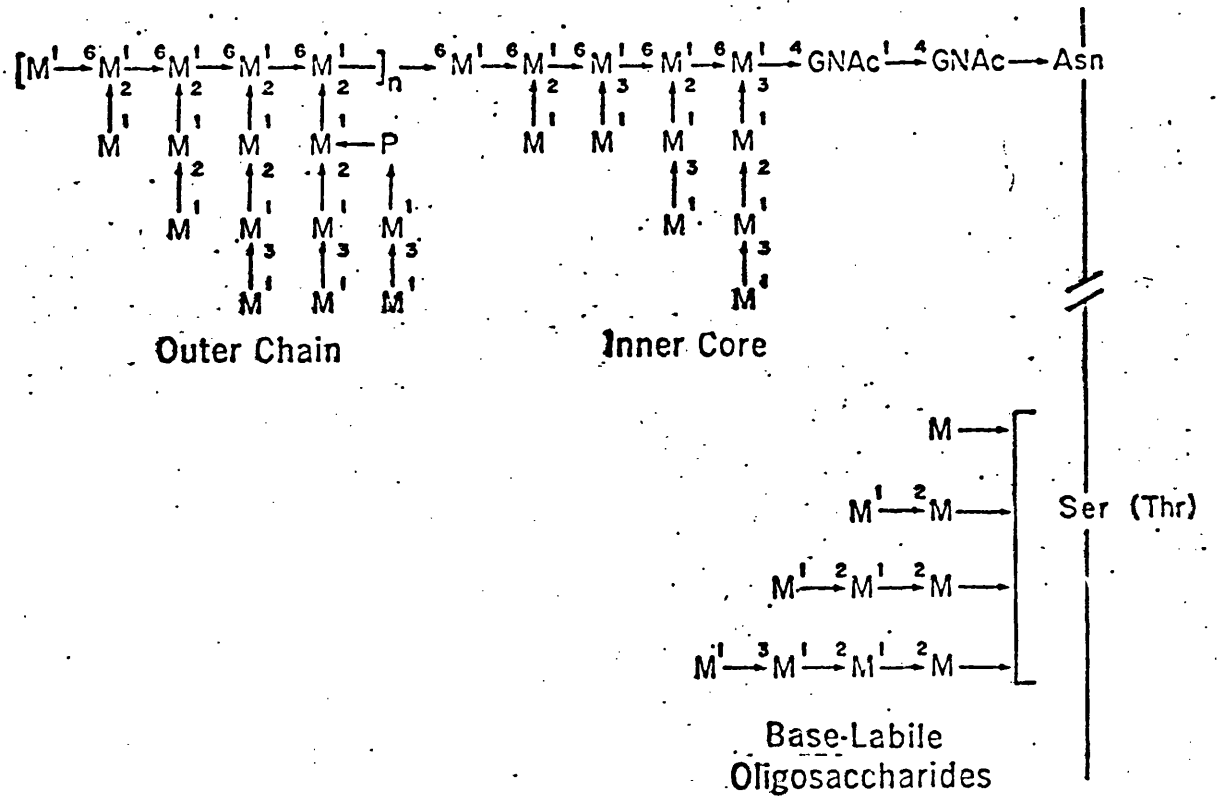


Figure 6 Schematic structure of baker's yeast mannan, from Ballou (1976). M indicates an α-D-mannopyranosyl residue.

In this structure, N-acetyl-D-glucosamine serves as the bridge between polysaccharide and protein where polymannose chains are linked to the protein by alkali-stable linkages.

Immunochemistry of Mannan.

Antisera obtained by injection of heat-killed yeast cells contains antibodies against various groupings on the mannan molecule (Suzuki, Sunayama & Saito, 1968; Ballou, 1970). The main antigenic determinatns of *Saccharomyces cerevisiae* mannan have been established as the α(1-2) and α(1-3)-linked side chains (Suzuki *et al.*, 1968; Suzuki & Sunayama, 1968). These workers also reported that the terminal (1-3)-linked α-D-mannopyranosyl residue seems to be more immunogenic than the (1-2)-linked residue. The α-D-mannopyranosylphosphate group in the mannan of some *Saccharomyces* strains is another important immunogenic group (Raschke & Ballou, 1971). However, Rosenfeld (1974) reported that the α(1-3)-mannobiosylphosphate group in *Saccharomyces cerevisiae* S288C mannan cannot be differentiated, immunochemically, from any other terminal α(1-3)-linked mannobiose residue. Antiserum obtained by injection of *Saccharomyces cerevisiae* cells that lack terminal α(1-3)-linked mannose has been found to be specific for the terminal α(1-2)-linked mannose, whereas that prepared against a mutant that does not add side chains to the backbone is specific for unsubstituted α(1-6)-linked mannose chains (Raschke *et al.*, 1973). From these results it is clear that

antisera of various specificities can be prepared and used to identify new or unknown determinants on the mannan of other yeast strains.

Glucosamine

The glucosamine content of the walls of *Saccharomyces cerevisiae* has been reported to be about 0.8 - 0.9% of the dry weight (Eddy,1958). Korn & Northcote (1960), who fractionated baker's yeast walls with ethylenediamine, concluded that only 9% of the apparent glucosamine content of the wall had solubility properties typical of chitin. However, Bacon *et al.* (1966) reported that 20% of the glucosamine content is present as insoluble chitin in baker's yeast walls. From the work of Houwink & Kreger (1953) and Bacon *et al.* (1966) it became evident that this insoluble chitin occurs in both bud-scar areas and non-scared tissue. Following earlier suggestion by Eddy (1958) and Korn & Northcote (1960), Sentandreu & Northcote (1968) obtained proof that the part of N-acetyl-D-glucosamine, which is not present as chitin, forms one type of linkage between mannan and protein.

Protein and Carbohydrate-Protein Complexes.

Protein: The protein content of baker's yeast walls was reported to be as high as 13% in the early literature (Northcote & Horne, 1952). This may be due to impurities in the cell-wall preparations. Many of the later studies showed that the protein

of baker's yeast walls is approximately 5 - 7% of the wall dry weight (Roelofsen, 1953; Falcone & Nickerson, 1956; Miller & Phaff, 1958; Buecher, 1968). A number of studies have been made on the amino-acid composition of the cell-wall protein. Kessler & Nickerson (1959), who obtained two fractions of glucomannan-protein from walls of *Saccharomyces cerevisiae*, made a detailed study of the amino-acid residues present. Their fraction I contained 17 different amino acids with glutamic acid (17.8% of the recovered amino acids) and aspartic acid (13.1%) in major proportions. Among the other amino acids leucine, lysine and alanine accounted for 9.1%, 8.1% and 6.9% respectively, while serine and threonine accounted for 4.1% and 5.0% respectively. The glucomannan-protein fraction II yielded 13 different amino acids. Lacking were proline, methionine, histidine and phenylalanine. In this protein, aspartic acid (31.1%), glutamic acid (9.2%), cysteic acid (8.4%), leucine (7.0%) and alanine (6.5%) were the most abundant amino acids, whereas serine and threonine accounted for 4.6% and 5.9%, respectively.

Mannan-Protein complexes: The presence of nitrogen, phosphorus and sulphur in preparations of mannan from baker's yeast walls has been reported by many workers (Northcote & Horne, 1952; Falcone & Nickerson, 1956; Nickerson & Falcone, 1956a,b). From their work, Falcone & Nickerson (1956) concluded that the mannan and protein are tightly bound and that the two components are present in a weight ratio of approximately 12:1. Kessler & Nickerson (1959), who studied two glucomannan-protein complexes

prepared from baker's yeast, believed that the highly acidic proteins of the wall are linked by carboxyl groups in acidic amino acids to the hydroxyl groups of the polysaccharide. However, since Northcote & Horne (1952) found an appreciable amount of protein in mannan extracted by hot alkali, the protein-polysaccharide linkages appear to be more complex than indicated by Kessler & Nickerson (1959). Korn & Northcote (1960), who fractionated baker's yeast walls with anhydrous ethylenediamine, obtained three fractions. Fraction A which was soluble in ethylenediamine and water consisted of a mannan-protein complex with 1.7% glucosamine and 0.3% phosphorus. Fraction B, which was soluble in ethylenediamine but insoluble in water was considered possibly to be a glucan-mannan-protein complex, with 0.8% glucosamine and 0.12% phosphorus. The third fraction (C), insoluble in ethylenediamine consisted of mannose, glucose, protein and glucosamine. From these results, Korn & Northcote (1960) suggested that non-chitinous glucosamine might well provide the link between protein and carbohydrate. Later Sentandreu and Northcote (1968) re-investigated the three fractions from ethylenediamine fractionation, after further purification by pronase digestion and fractionation on Sephadex columns, yielding corresponding fractions A2, B2 and C2. They found that fraction A2, on treatment with 0.1N NaOH yielded double bonded dehydroserine and dehydrothreonine together with mannose and oligosaccharides of mannose. From these results they suggested that the hydroxy amino acids were linked through

O-mannosyl bonds to mannose and small mannose oligosaccharides. These oligosaccharides included mannobioses linked by α(1-2), α(-3) and α(1-6) bonds (Sentandreu & Northcote, 1969). However, after this treatment with NaOH the large mannan molecule was still linked to the peptide. It was then found that aspartic acid was the only amino acid in the alkali treated mannan that occurred in equimolar amounts with glucosamine. From these results and periodate oxidation studies the second type of linkage between polysaccharide and peptide was suggested to be a nitrogen glycosyl bond between N-acetylglucosamine and aspartamide. Since the N-acetylglucosamine was not cleaved by periodate, it was thought that the linkage to mannan is through C-3 or C-4 or both This type of structure could accoumt for the high proportions of glucosamine not present as chitin in yeast walls. Later Cawley & Letters (1969) confirmed the results of Sentandreu & Northcote (1968). Much later Tarentino, Plummer and Maley (1974) reported that the majority of carbohydrate in crude mannans is attached to asparagine residues in the protein probably by way of di-N-acetyl-chitobiose units.

Cell-Wall Enzymes

A number of enzyme activities have been detected in walls of *Saccharomyces cerevisiae*. These enzymes are mainly hydrolases and enable the yeast to utilize substrates to which the plasma membrane is impermeable. Of these enzymes, invertase has been studied most widely. Neumann & Lampen (1967) prepared a highly

purified invertase from a *Saccharomyces* strain, which had a molecular weight of about 270,000 daltons. This was found to be a glycoprotein with about 50% mannan and 3% glucosamine. The protein part of the molecule contained a high proportion of aspartic acid, serine and threonine residues.

The Zeta Potential of Yeast

Early workers who studied the electrophoretic mobility of yeasts, in media containing brewer's wort, concluded that they are negatively charged, probably due to adsorbed colloids with an isolectric point around pH 4.5 (Lüers & Geys, 1922; Pfeiffer, 1932). However Hening & Ay (1938) and Kölbel(1947) found that, in the presence of buffering salts, various strains of yeasts were negatively charged throughout the pH range 2.5 - 7.0. Wiles (1951), who studied the electrophoretic mobility of washed *Saccharomyces cerevisiae* in buffer reported that the cells were negatively charged and that this is relatively independent of the pH value.

However Jansen & Mendlik (1951) detected a pronounced increase in mobility between pH 4.5 and 5.5. Eddy & Rudin (1958a), who studied the electrophoretic mobility of a number of *Saccharomyces* strains over the range pH 2.0 - 10.0, concluded that the zeta potential of yeasts is pH-dependent and is due to three types of groups in the surface layers. Groups of type A was postulated to carry a positive charge at pH values lower than 4 whilst groups of type B carry a negative charge at pH values above 4. These

groups are associated with the protein fraction of the cell wall and correspond to - NH_3^+ and $-COO^-$ groups. Groups of type C, which have a pK value less than about 2, correspond to the phosphodiester groups of the phosphomannan of the cell wall which carry a negative charge over a wide range of acidities. All three types of groups contribute towards the overall charge of the cell surface depending on the pH value of the suspending liquid and the yeast strain. Many workers have studied pH - electrophoretic mobility curves to obtain information about the surface layers of yeast and ascospores. (Eddy & Rudin, 1958a,b; Briley *et al.*,1970; Fisher, 1975).

Lipids

Various investigators have reported on the lipid content of isolated yeast cell walls, usually in terms of bound and free lipids, the latter being extractable by organic solvents without previous hydrolysis (Phaff, 1971). Masschelein (1959) found as much as 13.5% of the dry weights as lipid in walls of beer yeast after acid hydrolysis. Eddy (1958), on the other hand, found less than 2% total lipids in wall preparations from various *Saccharomyces* species. There is also great divergence in the reported values for nitrogen and phosphorus contents in isolated lipids by various authors, which could indicate different contents of phospholipids. Since in the washing and purification of yeast cell walls there is no assurance that the lipid-rich plasma membrane is completely removed, much of the reported variations in lipid contents is probably due to the extent of removal of these membranes.

The components in the lipid fraction of the yeast cell wall could be grouped into classes as mono-, di- and triglycerides, sterol esters, free fatty acids and phospholipids (Bianchi, 1967). The content of phospholipids is very low in brewer's yeast walls (Bianchi, 1967), but has been reported to represent 1% of the cell walls of baker's yeast (Suomalainen, Nurminen & Oura, 1967). After hydrolysis of the lipid, the main fatty acid components are palmitic and stearic acids, whilst acids with 10 or 12 carbon atoms in the chain are absent or low in content, (Griffin & MacWilliam, 1969).

Organization of the Yeast Cell Wall

Many attempts have been made to demonstrate different components of the yeast cell wall in particular strata by the use of ultra-thin sections in the electron microscope. The results are not highly definitive although with proper fixatives (such as permanganate) three layers could be recognized in cells with thick walls. It is now generally accepted that mannan-protein complexes form the outer region of the cell wall of *Saccharomyces* species. Because of an abundance of (1-2) and (1-3) linkages in yeast mannan the hydroxyl groups on C-3 and C-4 are unsubstituted. The latter are cleaved by periodate and the aldehyde formed stains intensively with leucofuchsin. Mundkur (1960), used this cytochemical method to demonstrate that mannan forms the outer layer of the wall and glucan which does not react with this reagent the inner layer. However Balcon *et al.* (1969) pointed out that β(1-6) glucan

discovered by them in cell walls of baker's yeast would also react with periodate-leucofuchsin and could have contributed to the intensely stained outer layer.

From the large amount of information provided by Northcote (1963), Phaff (1963), Nickerson (1963, 1964), Nagasaki *et al.* (1966) and many others, Lampen (1968) put forward a schematic representation of the structure of logarithmic phase *Saccharomyces cerevisiae* walls. He proposed that the outer layer of the wall is constructed of large mannan molecules held together by 1-6-phosphodiester bonds. This conclusion is supported by the detection of phosphodiester type groups on the cell-wall surface by Eddy & Rudin (1958a) who studied the electrophoretic mobility of yeast. Their pH-mobility curves indicated the contribution towards the mobility from groups of the phosphodiester type. Immunochemical studies (Hasenclever & Mitchell, 1964; Ballou, 1970) and experiments on concanavalin A binding by yeast (Tkacz, Cybulska & Lampen,1971) also supply proof for an outer layer of mannan in the yeast cell-wall. It is now understood however that the phosphodiester bonds do not hold polymannose chains together (Thieme & Ballou, 1971; Colonna & Lampen, 1974a).

Since mannan-protein enzymes are released by PR-factor (Nagasaki *et al.*, 1966) concurrently with free mannan,Lampen (1968) suggested that it is likely that these enzymes are held just below the outer mannan layer, either attached through their many small mannan tufts by additional mannose-phosphodiester bridges or simply held in place by hydrogen bonds or other hydrophilic forces,

The main mannan chains are now believed to be bound to asparagine in protein through N-acetyl-D-glucosamine (Sentandreu & Northcote, 1968) or by di-N-acetyl chitobiose units (Tarentino *et al.*, 1974) while the base-labile oligosaccharides are linked through O-mannosyl bonds to serine and threonine residues of the protein (Sentandreu and Northcote,1968). The location of mannan-protein enzymes just below the outer layer of the wall is consistent with the finding of Preiss (1958) who measured the sensitivity of invertase in thin films of yeast to low-voltage electron beams of increasing penetrating power. By this criterion, invertase was located 20 - 50% of the way through the yeast cell wall.

The release of mannan from intact cells by pR-factor usually continues for some time after invertase has been liberated (Nagasaki *et al.*, 1966). On the basis of this discovery, Lampen (1968) suggested that there may well be additional phosphodiester linked mannan below the enzyme layer; in fact the enzymes may be essentially surrounded by the mannan mesh.

Since the remaining mannan (about 20% of the total) and the glucan are released only very slowly by pR-factor, Lampen (1968) suggested that this material probably represented the glucoprotein fraction studied by Northcote (1963), Kessler & Nickerson (1959) and others, that can be released from purified walls by treatment with dilute alkali. This layer appears to be covalently linked to the glucan fibrils which constitute the rigid lattice that give the wall its characteristic shape.

The observation that thiol-containing reagents sensitize the

cell wall to the action of snail enzyme or other wall-digesting enzymes led Bacon *et al.* (1965) to propose that disulphide bonds link protein molecules in the wall so as to produce a lattice structure which provides additional strength. This concept is supported by Nickerson's earlier evidence (1963) that a protein disulphide reductase is necessary to render the local wall structure plastic before bud formation can occur. Since thiol-containing reagents also facilitate the action of PR-factor on walls, Lampen (1968) has suggested that their precise function will not be defined until studies can be carried out with pure preparations of the essential lytic enzymes. Most of the chitin in *Saccharomyces cerevisiae* walls has been located in the bud-scars (Bacon *et al.*, 1966; Bernan, 1968).

Although most of the data available up to date support the schematic structure of Lampen (1968) there is still no proof that the surface mannan completely covers the glucan. One way of testing this is by reacting yeast cells with glucan-specific antibodies. However glucan-specific antibodies do not appear to be formed by injection of rabbits with isolated glucan or cell-wall fragments (Ballou, 1976). It has also been reported that antigenic determinants that react with mannan-specific antibodies are detectable on the surface of *Saccharomyces cerevisiae* protoplasts (C.E. Ballou, unpublished results). This could indicate the presence of mannan in layers close to the cell membrane although other explanations are possible..

BINDING PROPERTIES OF THE YEAST CELL WALL

The numerous binding properties exhibited by the yeast cell wall could be broadly classified into two categories: (1) Binding of chemical compounds illustrated by cation binding and binding of non-ionic compounds such as proteins, silicones and hop constituents. (2) cell-cell binding illustrated by flocculation and sexual agglutination of certain yeasts.

The yeast cell wall is capable of binding a host of chemical compounds which include inorganic ions, organic compounds and organosilicon compounds. Binding of inorganic ions, mainly bivalent cations, has been studied in detail by Rothstein (1955), Rothstein & Hayes (1956), Mill (1964b), Lyons & Hough (1970a, b, 1971), Taylor & Orton (1973, 1975) and Stewart, Russell and Garrison (1975). Binding of hop substances from wort during fermentation was studied by many workers including Dixon (1967), and Dixon & Leach (1968) who reported that adsorption of hop substances onto yeast followed the classical pattern of Freundlich absorption isotherm. The only study on silicone binding is by Vernon & Rose (1976), polydimethylsiloxane being the compound studied. Binding of proteins by yeast is well illustrated by binding of antibody and concanavalin A. Binding of concanavalin A, which is a phytohaemagglutinin isolated from jack bean (*Canavalia ensiformis*) has been studied by Tkacz *et al.* (1971). Concanavalin A binds specifically to α-mannan of the cell wall and α-(1-2)-D-mannopyranosyl residues has been suggested as the specific

combining sites (So & Goldstein, 1968). The importance of bivalent cations to the concanavalin A-Polysaccharide interactions has been discussed by Agrawal & Goldstein (1968).

Sexual agglutination of *Hansenula wingei* has been studied in great detail by Brock (1959), Crandall & Brock (1968 a, b,c), Crandall & Caulton (1973) and Crandall, Lawrence & Saunders (1974). Sexual agglutination between opposite mating types of *Saccharomyces cerevisiae* also has been reported by Duntze, Mackay & Manney (1970).

SILICONES

The group of organosilicon compounds, known as silicones, has a multitude of industrial and commercial uses. The foundations of organosilicon chemistry were laid in the period 1899 to 1944 by Kipping who first prepared and examined silicones. In the course of this work he prepared silicones in oil and resin form, but could not realise the potential importance of these products. It was he who coined the name 'Silicone' as he thought these organosilicon compounds were analogous to ketones. However, it is now known that these are not monomeric compounds containing Si=O groupings as Kipping believed, but polymeric materials containing -Si-O-Si-linkages.

The first major use of silicones was in the form of grease, for coating spark plugs of aircraft engines in the Second World War. Later a host of silicone products were made with applications in industry, mainly as an insulating and water-proofing material, and in the manufacture of polishes. Now they are being used in food and pharmaceutical industries as well.

The most useful members of the family of linear polysiloxanes are the polydimethylsiloxane fluids, which have the structure shown in Figure 7.

Direct evidence of the structure of polysiloxanes is best obtained by the study of monemolecular films on the surfaces of liquids. The most general form of the linear molecule is a ball (Noll, 1968). It can coil up in this way owing to the

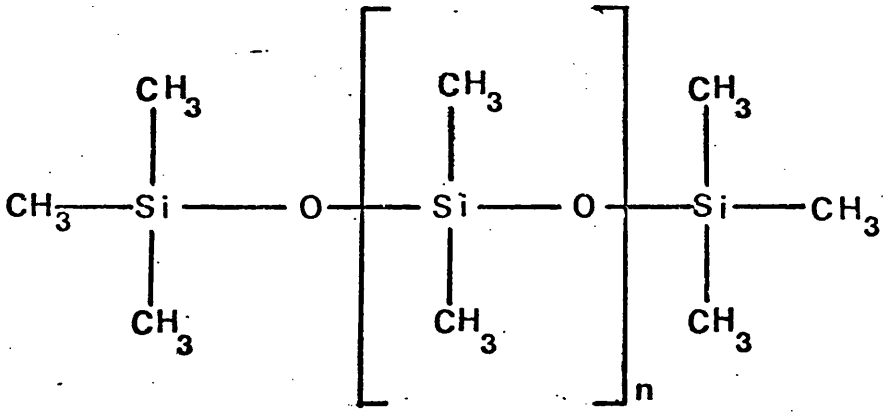


Figure 7. Structure of polydimethylsiloxane.

relatively large valence angle of the siloxane oxygen and the size of the silicon atoms, which results in the methyl groups being pushed so far from the Si-O-Si axis that free rotation around the siloxane bond becomes possible. It has been suggested that linear polydimethylsiloxanes assume 'the random coiled conformation' when dissolved in organic solvents (Noll, 1968). Much of the physical behaviour of this organosilicon polymer could be explained by considering that the siloxane chains take the form of a reversibly-coiled helix, as proposed by Pauling (1947), in which the ionic characters of the siloxane bonds are internally compensated. This idea of a helical structure is supported by

the work of Bridgman (1949), Ellison & Zisman (1956) and Banks (1957). These two theories may be regarded as alternative as regards explaining the bulk properties of silicone fluids.

Silicones are generally colourless, clear and tasteless. The fluids are highly compressible, hydrophobic and chemically inert, although they allow free passage of gases. Their chemical non-reactivity, low solubility in water and physiological inertness make them compatible with most drugs, foodstuffs and for some purposes biological systems (Levin, 1958). Their physical and chemical properties have been well documented (Baker, Barry and Hunter, 1946; Hurd, 1946; Patnode & Wilcock, 1946; Sauer & Mead, 1946; Bass, 1959). Silicone fluids are normally available in viscosities ranging from 0.65 cS to $2x10^5$ cS at 25^oC. The surface tension values of these increase from 15.9 dynes per cm. for a 0.65 cS fluid, to 20.5 dynes per cm. for a 20 cS fluid. In the 20 to 1000 cS grades the surface tension increases from 20.5 to 21.1 dynes per cm. Even high-viscosity fluids of polydimethylsiloxane have a lower surface tension than organic fluids such as benzene, ethanol or ethylene glycol. Low surface tension indicates high surface activity of these polymers. Jarvis (1966) has reported that surface viscosities of silicone fluids were below the limit of surface viscometers used and that this reflected the low intermolecular cohesion that exists between adjacent siloxane chains in a monolayer.

Silicones as Antifoams

The high surface activity of linear polysiloxanes led to their use as surface-active agents in the preparation of antifoams (Schwarz & Reid, 1964), for both aqueous and non-aqueous systems (Bhute, 1971). Depending on the systems in which they are used, silicone antifoams can be classified as follows: (1) agents effective in aqueous systems; (ii) agents effective in non-aqueous sytems; (iii) agents effective in miscellaneous systems. As far as fermentation industries are concerned the most important are those in the first category.

However, as practical antifoams, the neat silicone fluids are relatively ineffective, principally because of their high interfacial tension with water which is about 42 dynes per cm. (Evans & Hall, 1971). Their spreading coefficient is consequently low and they do not readily disperse in the foaming system. The most common method of application of silicones as antifoaming agents is in the form of an emulsion. The silicones are emulsified with suitable surfactants (Morel, 1958) and other ingredients which may be incorporated in the formulation to give an antifoam emulsion.

Compounded silicone antifoams usually contain finely divided silica (Evans & Hall, 1971), which markedly improve their antifoam action although the degree of improvement is highly dependent on the correct balance of particle size and hydrophilic character of silica. The role of silica is not completely understood although Ross (1967) suggested that it provides a polar surface

for water to enter and sub-divide large silicone droplets in the emulsion. Therefore emulsification and the presence of silica allow the silicone fluid to be in the form of small droplets in the antifoam.

The use of silicone antifoams in fermentations has occurred only recently, mainly due to the lack of suitable emulsifying agents. Sorbitan esters of fatty acids, their polyoxyethylene derivatives, glycol esters and glycerol esters, are nowadays generally accepted as efficient emulsifiers (Morel, 1958; Marshall & Williams, 1966). These are normally used in specific combinations to give the correct hydrophilic-lipophilic-balance which ensures the stability of the emulsion (Boyde, Parkinson & Sherman, 1972). The number of silicone formulations (with different emulsifying agents) which possess antifoaming ability is very great and their action as antifoams in industry has been discussed in detail (Currie,1953; Bhute, 1971). The use of silicones in industrial fermentations as antifoam agents has certainly been encouraged by the very low levels of toxicity that these polymers exhibit (Rowe, 1948). They seem to be unable to support microbial growth unlike other antifoams.

The use of silicones in industrial fermentations has been reported by Evans (1972), and in the antibiotic industry they have found a wide and varied use. Polydimethylsiloxane preparations (Köffler & Goldschmidt, 1950) with oleic acid when used as antifoaming agents in the production of penicillin resulted in greater oxygen transfer and higher yields of

penicillin. Similar observations have been reported for production of streptomycin (Coppock, 1950) and novobiocin (Soifer, 1966). Zaley *et al.* (1965) tested a large number of silicones as antifoaming agents in cultures of *Staphylococcus aureus* whose ability to form α-toxin is very sensitive to toxic factors. They found that a number of silicones were ideal as antifoaming agents in these fermentations. Furthermore they found for a whole range of fermentations that, even if the quantity of silicone antifoam needed for desired antifoaming action was exceeded, there was no adverse effect on the metabolism of the bacteria. Kristapsons (1972) employed silicones as antifoaming agents in amino-acid producing fermentations. *Candida utilis* (an L-tryptophan producer) and *Brevibacterium* species 22 (an L-lysine producer) were tested and found to be unaffected by the presence of silicone. *Pseudobacterium lacticum* 392 and *Pseudomonas liquefaciens* 399 showed increased amino-acid production when silicones were tested, against a control in which sunflower oil antifoam was used. Kristapsons (1972) suggested that increased oxygen-transfer rates due to decreased foam production may be responsible.

The effect of silicones on wine production has been studied by Ruiz (1964, 1968, 1970). He found that addition of silicone antifoams prevented foam formation at the beginning of the fermentation and that the resulting wine had a higher concentration of alcohol. Prevention of foaming in this way also led to the disappearance of bacteria associated with the foams, thereby

yielding less acidic wines.

Formulations made up of polydimethylsiloxane along with other suitable ingredients mixed in refined petroleum oils has been employed in the form of water-in-oil emulsions to prevent foaming in yeast and sugar industries. The work of Mel'tser & Kuramshin (1958) has shown that the use of silicones in large-scale industrial production of yeast gave increased yields and a higher quality of yeast when compared with the use of other antifoam agents. It also was reported that there were no adverse effects on the yeast either metabolically or as regards the viability.

The major benefits of employing a silicone antifoam in brewery fermentations has recently been reported (J.I. Evans, unpublished communication) and these features demonstrate the contribution which silicones can make to plant economics and efficiency. They include: (i) efficient control of foam which allows an increase in fermenter capacity, (ii) better utilization of hop material, (iii) no effect on the yeast, (iv) good head retention of the final beer, (v) easy cleaning of fermenter vessels due to the decrease in foam adhering to the sides and (vi) no silicone remaining in the final beer after removal of the yeast and treatment of the beer with absorbants and after filtration.

Binding of Silicone by *Saccharomyces cerevisiae*

Binding of polydimethylsiloxane (PDS) on to *Saccharomyces cerevisiae* NCYC 366 has been studied by Vernon & Rose (1976). They found that binding of PDS has no effect on the growth or

respiratory activity of the yeast, and the binding itself is not affected by the temperature or the pH value of the medium. Isolated walls bound PDS faster than intact organisms. Incubation of walls saturated with PDS in the presence of buffered (pH 8.0) EDTA (10 or 100 mM) caused the release of PDS from the walls. However, incubation of these saturated walls in the presence of 8M urea did not lead to any release of PDS.

FLOCCULATION OF YEAST

Flocculence is one of the most important properties of brewer's yeast. This is well reflected by the many review articles in literature and more especially within the brewing-oriented journals. The term 'flocculence' in brewing technology refers to the agglomeration of yeast cells at the end of fermentation. However, it is now used in a more general sense to describe the agglomeration of yeast cells suspended in a suitable medium or buffer solution which may contain calcium ions. The mechanism of flocculation is still not completely understood. However it is generally believed that the determinants of flocculence reside in the yeast cell wall and the phenomenon is manifested predominantly in the stationary phase of growth. Environmental factors too appear to play an important part in yeast flocculation.

Various aspects of yeast flocculation have been reviewed by many workers. Of the general surveys to be found, most comprehensive are by Burns (1937), Comrie (1952), Jansen (1958), Morris (1966), Rainbow (1966, 1970), Windisch (1968) and Geilenkotten & Nyns (1970a). The genetical aspects of flocculation have been reviewed by Thorne (1952), Gilliland (1957, 1958, 1971b) while its possible application to yeast classification is reviewed by de Becze (1962), Gilliland (1971c) and Stewart *et al.* (1975). Articles by Rose (1963), Lyons & Hough (1970c), McWilliams (1970), Geilenkotten & Nyns (1971), Stewart (1975) and Stewart *et al.* (1975), discuss the relationship between flocculation and cell-wall structure.

Its role in brewery fermentations is reviewed by Cook (1963, 1969) and Kleyn & Hough (1971). The following account aims to summarize these reviews and supplement where appropriate, with more emphasis on the physicochemical and biochemical aspects of yeast flocculation.

Importance of Flocculation in Brewing

The phenomenon of flocculation is important to the brewer mainly because it determines the extent of fermentation (the degree of attenuation) of his worts, by controlling the extent of contact between yeast cells and wort nutrients. Highly flocculent yeasts separate early from suspension and consequently tend to produce less well attenuated, sweeter beers with the absence of yeasty flavours. However they are possibly more susceptible to contamination by spoilage organisms. Conversely, poorly flocculating (powdery) yeasts carry on the fermentation for a longer period of time, yielding well attenuated, microbiologically stable beers. However such beers too may be undesirable due to low clarification and possibly unpleasant flavours. Therefore those strains with intermediate powers of flocculation are generally selected for brewing. However, in batch fermentations the flocculation aspect is somewhat less important, as the yeast could be centrifuged off when required. But in continuous tower fermentations a plug of flocculent yeast is said to be essential.

Classification of FLocculation

The flocculence characterisitics of brewer's yeasts have been discussed and classified by many workers including Hough (1957),

de Becze (1962), Emeis (1970), Gilliland (1951, 1971a,b,c), Thorne (1972) and Stewart *et al.* (1975).

Gilliland (1951) categorized top-fermenting brewing yeasts into four groups according to their flocculence. Those of class I are completely dispersed at all stages of the fermentation, while class II yeasts are initially completely dispersed, forming small loose clumps towards the end of the fermentation, and therefore most valuable as brewing strains. Class III strains also separate out late in the fermentation but form large dense masses. Class IV yeasts begin to flocculate soon after the start of the fermentation because of the failure of newly forming cells to separate.

Hough (1957) used flocculence characteristics to classify pitching yeast into five categories, as follows: (a) form films of cells at the air-liquid surface of aqueous suspensions of yeast (head formation); (b) form aggregates in calcium chloride solutions buffered at pH 3.5 which (i) dispersed on addition of maltose, and (ii) failed to aggregate when ethanol was added; (c) form aggregates in calcium chloride solutions buffered at pH 5.0; (d) form aggregates in calcium chloride solutions when an appropriate second strain was present (co-flocculation); (e) form chains of cells in malt-extract liquid medium (chain formation).

Campbell (1967) demonstrated that flocculent non-flocculent strains of the same species of yeast were antigenically identical although the relative amounts of mannan in the cell wall varied

with flocculence. He suggested that the ratio of antigenically active material could possibly be employed as a basis for classification at least for certain yeast.

Co-flocculent strains have been further investigated by Stewart (1972) and Stewart & Garrison (1972). Stewart *et al.* (1975) classified ale yeast into five categories, on their flocculation characteristics, whereas in lager strains they recognised only two categories on the same basis. The five categories of ale strains are:

(1) Non-flocculent strains.
(2) Co-flocculent strains.
(3) Strains flocculent on their own after growth in wort, but non-flocculent when grown in buffered defined medium of glucose and ammonium salts (pH 4.5).
(4) Strains flocculent on their own after grown in either wort or glucose-ammonium salts medium.
(5) Chain-forming yeasts.

The two categories of lager strains are as follows:

(1) Non-flocculent strains.
(2) Strains flocculent on their own even when grown in a defined medium. No wort inducer is necessary in the growth medium.

Measurement of Flocculation

The most common method used to estimate the degree of flocculation of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* is to measure the sedimentation rate of cells suspended in a synthetic medium or a suitable buffer solution. Exceptions to this are the methods used by Jonas, Briess & Minek (1944) and Jansen & Mendlik (1951, 1953) where electrical properties of the yeast cell surface were used.

Nielsen (1937) measured the sedimentation rate by estimating the proportions of yeast originally in the upper 75% of a suspension which passed into the lower 25% in each minute. The amount of yeast was estimated by evaporating samples of the suspension to dryness at 105°C. In the same year, Burns (1937) described a method in which he used a yeast suspension of known concentration in sodium acetate buffer (pH 4.6) to measure the sedimentation rate. The suspension (10 ml) was dispensed into 15 ml. graduated, tapered centrifuge tubes and the volume of the settled, flocculated cells at the bottom was noted after a definite interval of time. This method,with minor modifications, is still used for rapid estimation of yeast flocculence.

Jonas *et al.* (1944) attempted to relate the electrical conductivity of yeast cells to the determination of their flocculence potential and Jansen & Mendlik (1951, 1953) investigated flocculence using a micro-electrophoretic method.

An attempt to calculate the 'sedimentation constant' on the basis of that the logarithmic value of light absorption in

yeast cell suspensions exhibited a linear relationship to time was made by Hartong (1951), while Gilliland (1951) used a turbidometric method to follow sedimentation of yeast cells in growth medium.

Helm, Nohr & Thorne (1953), who modified the method of Burns (1937), recommended that the water used for washing and suspending yeast should contain 500 p.p.m. $CaSO_4$, since they confirmed that calcium ions are necessary for the expression of the flocculence characteristics of yeast. Kato & Nishikawa (1957) further modified the method by using a photometric technique to determine the amount of yeast in the upper 80% and lower 20% of the suspension which had been allowed to stand for 5 min at 20°C. From these values they calculated the 'sedimentation percentage' which gave a measure of the flocculence. Woof (1962) produced so-called 'sedimentation curves' by plotting the cell count against time, for a number of different yeasts in beer.

In more recent methods, the rate of sedimentation of yeast is followed by measuring the drop in the absorbance with time of a suspension of yeast in a buffer solution or a synthetic medium, with or without added calcium ions (Chester, 1963; Mill, 1964a; Ginterova & Janotkova, 1965; Greenshields, *et al.*, 1972). Mill (1964a) calculated the 'sedimentation rate' of yeasts by using a method in which he measured the drop in absorbance with time of a suspension of yeast (about 4.0 mg dry weight per ml) in sodium acetate buffer (pH 4.6) containing 0.1% (w/v) $CaCl_2$. The absorbance readings were then converted to apparent dry weight

of cells in the light path of the instrument and the steepest negative slope of the graph, obtained by plotting dry weight values against time, was taken as the 'sedimentation rate' which was expressed as µg dry weight per ml. per min. However, it is by no means certain that the sedimentation rates exactly parallel the aggregation powers of yeast cells. But the sedimentation rates obtained in this way give a measure of the initial rate of flocculation, which could be reproduced with reasonable accuracy. On a similar theme, Greenshields *et al.* (1972) used an automatic recording spectrophotometer to obtain initial flocculation velocities for sedimenting yeast taken from tower fermentation systems.

A comparatively simple method of grading floc size in a visual manner, where yeast cells are suspended in a buffer solution, has been used by Baker & Kirsop (1972). This method appears to be a reasonably rapid and useful flocculation test.

Taylor & Orton (1975) described a photometric method for measuring 'flocculation intensity' (F) of a yeast suspended in a buffer solution of a particular ionic strength, containing Ca^{2+} ions. The value of F was calculated from the equation: $F=I_{28}/I_m-1$, where I_{28} is the relative transmission of light at the steady state ($28^{\circ}C$) of the suspension and I_m is that above the deflocculation temperature, denoted by T_f.

Mechanism of Flocculation

As previously stated, although the importance of flocculation in brewing technology is quite apparent, the mechanism of the

process is still not completely understood. The subject has been reviewed by Comrie (1952), Jansen (1958), Rainbow (1966, 1970), Geilenkotten & Nyns (1971), Stewart *et al.* (1975) and Stewart (1975).

(a) Genetics of flocculation

There is good evidence that flocculence is genetically controlled in *Saccharomyces cerevisiae*. Thorne (1951a,b) crossed five strains of top-fermenting yeast by the method of Winge & Lausten (1937, 1938) and found that the flocculent parent strains gave rise to flocculent offspring, whereas the offspring of non-flocculent parents were also non-flocculent. However, the hybridization of flocculent strains with non-flocculent strains either failed to produce spores or the occasional spore failed to germinate. Thorne circumvented the problem by crossing a baker's yeast with a brewer's yeast, which gave rise to a hybrid that produced viable spores. Observations of their offspring showed the property of flocculence to be under genetic control and the gene controlling flocculence to be dominant over the gene for non-flocculence. According to Thorne (1951a,b) the genetic mechanism comprises at least three independent genes, the presence of any one sufficing to confer flocculating properties on that yeast strain.

However, in the same year, Gilliland (1951) obtained heterozygotes by the method of Lindegren & Lindegren (1943) of two strains of *Saccharomyces cerevisiae* which differed only in their flocculation properties and belonged to class I and IV

(Gilliland, 1951) respectively. From the studies made on their descendents, he confirmed that flocculence is a hereditary property, but controlled by only a single gene. Further, from his studies, Thorne (1951a,b) concluded that mutation rate from flocculence to non-flocculence is high. Clayton, Howard & Martin (1972) concluded from their experiments involving an extensive hybridization programme, that flocculence may well not be a dominant character and that the genetical basis of the phenomenon was probably very complex. Recently, Lewis (1974) idenfified three genes for flocculence, two dominant (Flo 1 and Flo 2) and one recessive gene (Flo 3). The presence of only one being necessary for the flocculence phenotype to be expressed. Lewis (1974) is also of the opinion that there is in many cases an influence of additional modifier genes or cytoplasmic genetic factors.

(b) Effect of environment on flocculation

Although the manner in which environmental factors influence flocculation is still to be adequately explained, it is established that certain chemical and physical properties of the environment must fall within certain limits for aggregation of yeast cells to occur.

Stockhausen (1927), who studied the effect of the pH value of the medium on flocculation, reported that an increase in the hydrogen ion concentration caused the electrical charge on the cell surface to diminish. There was an increasing tendency towards flocculation which reached its maximum at pH 3.8, when the cells

were electrically neutral. Hartong (1951) continuing on the same theme showed that flocculation occurred when the pH value of the fermentation medium had almost reached its final value, at which the surface charge of the cell had decreased to a minimum.

Gilliland (1955) reviewed the influence of environmental conditions on yeast flocculation , and included the effects of temperature, aeration, acidity, electric charge and the presence of fermentable and non-fermentable sugars and cellular metabolic products in the medium. Fermentable sugars are reported to prevent flocculation by many workers (Lindquist, 1953; Eddy, 1955a; Akin & Krabbe, 1966) while proteins are claimed to cause flocculation in some cases (St Johnston, 1953) and prevent flocculation in others (Lindquist, 1953; Kijima, 1954). However, since flocculation can occur in defined medium containing glucose and ammonium salts, Eddy (1955b) stated that complex wort constituents do not necessarily play a part in flocculation. But recently, it has been reported that peptide material present in wort can induce flocculation and co-flocculation (Stewart *et al.*, 1975). This peptide material has been isolated and characterised by Stewart *et al.* (1975) and its structure has been compared and found similar to that reported for the α-factor involved in sexual agglutination of haploid α and a cells of *Saccharomyces cerevisiae.*

Regarding the temperature of the medium, it has been long recognized that the lower the temperature the greater the flocculation. Mill (1964b) reported that raising the temperature of the medium to 50 - 60°C caused complete deflocculation of flocculent yeast.

Taylor & Orton (1975) used the term 'floc dissociation temperature' (T_f) to denote the temperature at which complete deflocculation of a flocculent suspension of yeast occurred.

Mill (1964b) reported that Ca^{2+} ions are essential for flocculation and, at a concentration of 0.2 m mol per litre, almost complete flocculation would occur. The effect of Ca^{2+} ions on flocculation is antagonized by monovalent ions such as Na^{+}. Taylor & Orton (1973) found that the alkaline-earth metals strontium and barium, as well as calcium-complexing substances inhibit flocculation in strains of *Saccharomyces cerevisiae*. Later they (Taylor & Orton, 1975) confirmed that a requirement for small amounts of Ca^{2+} is essential for flocculation as reported by Mill (1964b), and specific since Mg^{2+} or other bivalent metals cannot replace it. However they also stated that the concentration of free Ca^{2+} in solution required for flocculation is in the region of 10^{-8} moles per litre and is much less than reported by Mill (1964b). The involvement of Ca^{2+} in flocculation was explained by Mill (1964b) and earlier by Harris (1959) who suggested the formation of salt bridges where a Ca^{2+} ion joins two carboxyl groups at the surface of two yeast cells, the structure thus formed being stabilized by hydrogen-bonding between complementary hydrogen and hydroxyl groups in the surface. In support of such a theory, Mill (1964b) demonstrated that treatment of potentially flocculent yeast with 1,2 epoxypropane (an agent for esterifying carboxyl groups) causes deflocculation, due to blocking of carboxyl groups. He also showed that the treatment with urea which decreases hydrogen

bonding caused deflocculation of potentially flocculent yeasts.

However, Lyons & Hough (1970a, b, 1971) who further studied the Ca^{2+} cross-bridging hypothesis of Mill (1964b), suggested that it is phosphate groups in the cell wall and not carboxyl groups that are joined through Ca^{2+} bridges.. In support of this theory they found that walls of flocculent strains of *Saccharomyces cerevisiae*, which contained more phosphorus on the wall surface than non-flocculent strains, bound twice as much calcium compared to walls of non-flocculent strains. Furthermore they found that esterification of the carboxyl groups of the wall surface with 1,2 epoxypropane caused a drop of only 20% in the calcium binding capacity of the wall indicating that the part played by carboxyl groups is minor but significant.

Recently, Stewart *et al.* (1975) reported that studies on the adsorption of Ca^{2+} ions by the cell wall of *Saccharomyces cerevisiae* and *carlsbergensis* failed to reveal any significant differences in total uptake between flocculent and non-flocculent strains. They suggested that the contribution of calcium ions to flocculation is not the absolute amount of this ion adsorbed by the yeast cell wall but rather the stereo-specific manner by which it is bound, i.e. its position relative to the three dimensional structure of the yeast cell wall.

Another property of the medium which has a significant effect on flocculation is the dielectric constant. Mill (1964b) showed that an increase in the dielectric constant of the medium causes deflocculation. Addition of organic solvents such as

ethanol, methanol, iso-propanol, acetone or dioxane, which decreased the dielectric constant of the medium, caused non-flocculent yeast to flocculate.

Recently, Taylor & Orton (1975) who further studied the effect of the environment on flocculation reported that the ionic strength of the medium plays an important part and that the optimum ionic strength for flocculation, which depends on the yeast strain, lies in the range 0.005 - 0.05.

(c) Physico-chemical aspects of flocculation

There is evidence that most brewer's yeasts behave as negatively charged colloids in aqueous suspension at the pH value of worts and beers (pH 3.8 - 5.6). At more acid pH values, reversal of charge may take place depending on the strain. Although both flocculent and non-flocculent yeasts behave in this way, the former are believed to carry the lower charge (Jansen & Mendlik, 1951).

Flocculated yeasts are deflocculated by washing in distilled water or dilute solutions of calcium-chelating agents such as EDTA, and reflocculated by addition of divalent or polyvalent cations, especially Ca^{2+}. A possible explanation of these observations is that, when the charge on the cells reaches a certain minimum enabling their mutual repulsion to become insignificant, they may flocculate under the action of other forces. The observed effects of pH value and divalent and polyvalent cations are consistent with such a theory (Rainbow, 1966).

However, Eddy & Rudin (1958b) who studied the electrophoretic mobility of strains of *Saccharomyces cerevisiae* and *carlsbergensis* found that this was independent of their flocculation characteristics. These results indicate that flocculence and surface charge of the yeast cell are not interrelated. However this does not exclude the possibility of charged groups of the surface taking an active part in flocculation.

(d) Biochemical aspects of flocculation

Accounts of the possible mechanism of yeast flocculation based on the involvement of cell-wall components are abundant in the literature. The structural configuration of the cell-wall components appear to play a very important part in these considerations.

Eddy & Rudin (1958c) found that treatment of isolated walls of *Saccharomyces cerevisiae* with papain destroyed flocculence. They attributed this to the loss of a mannan-protein complex from the cell wall, which was the principal product made soluble by the papain treatment. Two types of chemical groups, each probably carried by the mannan-protein complex, were postulated to be involved in the flocculation mechanism at least in those strains which aggregate only in pairs (co-flocculence).

In the following year, Harris (1959) associated flocculation with the termination of fermentation and thus with the stationary phase of growth. The requirement for bivalent cations, especially Ca^{2+} and Mg^{2+} in the cellular environment led to the suggestion that salt bridges are formed between two yeast cells, in which metal ions are held by two carboxylate ions and two co-ordinate

links from suitably placed donor groups one from each separate yeast cell.

Masschelein *et al.* (1963) claimed that the period of deflocculation coincides with a synthesis of mannan and accordingly that this polysaccharide acts as a regulator of the intensity of flocculation, by masking more or less completely the active groups of the specific fraction carrying the flocculence character. Their comparative study of a flocculent and a powdery strain of *Saccharomyces cerevisiae*, in the presence of 2,4 dinitrophenol, showed that the flocculent strain possessed an intracellular biochemical mechanism which allowed rapid utilization of mannan. Chromatographic analysis of protein hydrolysates from walls of cells harvested at the end of fermentation were characterized by an increase of 30 - 40% in total amino acids. Taking into account the parallel decrease in mannan content at the end of fermentation they concluded that it is essentially the ratio of mannan to protein that determines the physiological state of the cell. Jeunehomme-Ramos, Castiau & Masschelein (1964) assumed that flocculence was a cyclic phenomenon, the duration of the phases depending on the rates of synthesis and degradation of mannan.

At this period many theories began to flood the literature on the mechanism of flocculation . Mill (1964a) suggested somewhat contradicting the theory put forward by Masschelein *et al.* (1963), that maintenance of non-flocculence was dependent upon the presence in the wall of a nitrogenous compound and that

potential flocculence developed when this compound was not synthesized at a rate sufficient to maintain a high enough level in the wall to mask a certain mannan constituent conferring flocculence. Mill (1964b) reported the involvement of carboxyl groups carried by acidic amino-acid residues (aspartic and glutamic acids) in the protein, since esterification of these groups with 1,2 epoxypropane destroyed the ability of cells to aggregate. He also put forward a hypothesis, similar to that of Harris (1959), in which flocculated cells were thought to be linked by salt bridges, where two carboxyl groups on the surface of different cells are linked together through a Ca^{2+} ion bridge. Furthermore this structure was assumed to be stablized by hydrogen-bonds. Kijima (1964),by treatment of yeast cells with dilute alkaline solutions at room temperature, obtained two materials flocculent and non-flocculent, and their behaviour in various media was similar to that of the yeast itself. The important difference in the composition of these two materials was in the polysaccharide content, which was about 6% of the protein content in the flocculent material and 36% in the non-flocculent material. He suggested that the origin of the non-flocculent character of intact powdery yeasts may possibly be a protein-polysaccharide complex on the cell surface. The polysaccharide when present would mask the functional groups of the protein which otherwise would make yeast cells flocculent especially near pH 4.5.

The phosphomannan component of the cell wall began to assume

greater significance at this stage. Again, Mill (1966) demonstrated that the degree of phosphorylation of mannan varied from one phosphate to 19 mannose residues in non-flocculent yeast to one phosphate to 13 mannose residues in flocculent yeast. In the following year, Ito (1967a) found that the content of acid-soluble phosphorus compounds in the cell wall of flocculent yeasts was higher than that of non-flocculent yeasts. In another publication in the same year, he (Ito, 1967c) considered that negative charges on *Saccharomyces cerevisiae* are generated by phosphate groups located near the surface of the cell. He suggested that, when the activity of energy metabolism of cells is vigorous, the negative charge density on the surface is high and the yeast cells resist flocculation. When the activity of energy metabolism of cells is decreased their negative charge density falls and the yeast cells flocculate. Ito (1967b) went on to conclude that the fundamental substance governing flocculation is the so-called 'zymocasein' fraction of nucleoprotein associated with the plasma membrane. Furthermore he (Ito, 1967d) stated that adsorption of tannin complexes formed by wort protein onto the cell surface greatly increased the flocculating power of a yeast.

In a series of papers, Lyons & Hough (1970a, b, 1971) discussed the role played by phosphomannan in calcium binding and how this correlates with yeast flocculation. They showed that walls from flocculent strains of *Saccharomyces cerevisiae* have higher contents of phosphorus in the outer layers and bind twice as much

calcium as walls from non-flocculent strains. Furthermore they suggested the involvement of phosphate groups of phosphomannan in the formation of cross-bridges through Ca^{2+} ions during flocculation. They failed to find a correlation between the sizes of mannan side-chains or acidic amino-acid contents in the walls and flocculation, and deduced that the capacity of cells to flocculate by cross-bridging was proportional to the number of phosphate groupings present in the outer layers of phosphomannan-protein of the cell-wall.

Griffin & MacWilliam (1969) found that the increase in the wall content of yeast cells as they pass to the stationary phase was greater in flocculent strains than in non-flocculent strains. They also studied the nitrogen, phosphate and carbohydrate contents of the wall and failed to find a significant trend in any of the components which could be related to flocculent or non-flocculent behaviour. This endorsed an earlier report by Anderson (1967) who had advocated a more enzymic approach to the subject.

Cawley & Ballou (1972) obtained evidence for two structurally and antigenically different *Saccharomyces cerevisiae* cell-wall mannans, but comparison between several flocculent and non-flocculent strains failed to show a clear-cut difference in the mannan structure or in the distribution of phosphomannan. The absence of such differences led them to conclude that agglutination of yeast cells could not be explained on the basis of salt-bridges alone. There would thus appear to have arisen two distinct factions here.

Firstly, there are those who advocate a purely chemical theory which explains flocculation as due to salt-bridges formed by charged groups of the cell wall through Ca^{2+} ions and further stabilized by links of the hydrogen-bond type. Secondly, there are those who, while not necessarily dismissing this hypothesis, envisage a much more complex mechanism perhaps involving several different interactions supplemented by the salt-bridges.

Geilenkotten & Nyns (1970b) were able to effect the flocculation of a *Saccharomyces cerevisiae* strain immediately at the outset of its stationary phase of growth by adding a glucanase-rich snail juice enzyme preparation to the medium. Subsequently in a B.I.R.F. Annual Report (J. Inst. Brew., biochemistry section, 1972) it was stated that examination of a wide range of yeasts, first by attack with enzymes from snail gut followed by coupling with fluorescein isothiocyanate, showed that the enzymic treatment altered the sedimentation rate, as judged by the 'Helm test', of exponential and stationary phase cells of many non-flocculent yeasts but changed the rate only of exponential phase cells of flocculent yeasts. The report also stated that removal of wall polysaccharides (chiefly mannan), with 0.05N sodium hydroxide brought about loss of flocculence thereby confirming the connection of this polysaccaride with flocculence.

Williams & Wiseman (1973) claimed that loss of flocculating ability of yeast coincided with the release of 70% of the mannoprotein enzymes, acid phosphatase and invertase, from the cell wall. They concluded that the role played by these enzymes in

flocculation is to control phosphate-Ca^{2+} interactions.

Work carried out by Stewart *et al.* (1973) resulted in a return to the notion of protein carboxyl groups being responsible for the cell surface's role in flocculation by involving Ca^{2+} in the medium. They found this mechanism to operate at least in certain co-flocculating strains. Stewart *et al.* (1975) and Day, Poon & Stewart (1975) reported that electron microscopic examinations of ether-washed yeast cells revealed that, whereas the cells of a non-flocculent culture appeared to be free from extra-cellular projections, cells from flocculent cultures were covered with an extensive layer of spicules or hair-like protuberances. They also stated that observations made on ale strains, which are flocculent when grown in wort medium but non-flocculent when grown in glucose-ammonium salts medium, showed that the cells do possess the hair-like projections when grown in wort medium but not when grown in glucose-ammonium salts medium.

Finally Fisher (1975), who studied the electrophoretic mobility-pH patterns of flocculent and non-flocculent strains of *Saccharomyces cerevisiae*, observed a pH-dependent re-arrangement of an underlying surface protein layer in the flocculent strain NCYC 1109. However, he could not obtain any evidence of a similar re-arrangement with other flocculent strains.

The study reported in this thesis was undertaken in order to investigate some binding properties of the cell wall of *Saccharomyces cerevisiae* with special reference to binding of polydimethylsiloxane and cell-cell binding (flocculation). In the case of PDS binding, some factors affecting the binding, cell-wall components responsible for the binding and the effect of PDS binding on some properties exhibited by the cell wall were investigated. In the case of flocculation, the effect of removal of phosphorus from the phosphomannan of the cell wall on the rate of sedimentation, and some surface properties were studied.

METHODS AND MATERIALS

CULTURAL METHODS

Organisms

In the study of polydimethylsiloxane (PDS) binding by yeast, the strain used was *Saccharomyces cerevisiae* NCYC 366. The study on flocculation was carried out using four strains of *Saccharomyces cerevisiae;* namely NCYC 366, NCYC 1004, NCYC 1005, NCYC 1063. The first two of these four strains were of the non-flocculent type and the other two of the flocculent type, as measured using the technique described by Mill (1964a).

Stock cultures of organisms were maintained on slopes containing 0.3% (w/v) malt extract (Munton and Fison Ltd., Stowmarket), 0.3% (w/v) yeast extract, 0.5% (w/v) mycological peptone, 1.0% (w/v) glucose, and 2.0% (w/v) agar; (MYGP medium, Wickerham, 1951), which were sterilized by autoclaving at 15 lb/in^2 (10.34×10^4 Pa) for 15 min. Slope cultures were transferred at monthly intervals. Four cultures of organisms were used in weekly succession. Two subcultures were made on each occasion. After 48 h incubation at 30°C, one culture was used for routine innoculation and the other was stored at 4°C for future subculturing. The purity of organisms was checked at intervals by plating out.

Experimental Media

Batch cultures of *Saccharomyces cerevisiae* NCYC 366 for the study of PDS binding were grown in a medium (pH 4.5) which had the following composition in g per litre of distilled water:

$(NH_4)_2SO_4$	3.0
KH_2PO_4	3.0
$MgSO_4.7H_2O$	0.25
$CaCl_2\ 2H_2O$	0.25
Glucose	20.0
Yeast extract	1.0

The medium was autoclaved at 10 lb/in ^{2} ($6.8_9 \times 10^4$ Pa) for 10 min.

For experiments on flocculation, organisms were grown in MYGP medium (pH 5.0; Wickerham, 1951). This had the following composition in g per litre of distilled water:

Malt extract (Munton and Fison Ltd.,Stowmarket)	3.0
Yeast extract	3.0
Mycological peptone	5.0
Glucose	10.0

The medium was autoclaved at 15 lb/in^2 (10.34×10^4 Pa) for 15 min.

Experimental Cultures

Organisms were grown batchwise in one-litre portions of medium in two-litre round flat-bottomed flasks. Cultures were incubated at 30°C, with stirring by means of the apparatus described by Patching & Rose (1970). To grow organisms for PDS binding studies one-litre batches of medium were innoculated with a portion, from a 24 h culture grown in the same medium,containing approximately 1.0 mg dry weight equivalent of organisms. Cultures were incubated overnight at 30°C and harvested in the mid-exponential

phase of growth, when a cell concentration of 0.22 - 0.25 mg dry weight equivalent per ml had been reached.

To grow organisms for flocculation experiments, one-litre batches of medium were innoculated with a portion from a 24 h culture grown in MYGP medium, containing approximately 10.0 mg dry weight equivalent of organisms. The cultures were incubated at 30°C and harvested in the stationary phase of growth when a cell concentration of approximately 1.3 - 1.5 mg dry weight equivalent per ml had been reached.

Organisms were harvested by centrifuging at 0°C in an MSE High Speed 18 centrifuge, at 2000 g. Organisms required for experiments other than on flocculation were washed in 0.1M-KH_2PO_4 buffer (pH 4.5) and used immediately. Organisms required for flocculation studies were washed twice in water, freeze-dried and stored at 4°C over silica-gel in a vacuum desiccator.

ANTIFOAM PREPARATIONS

Antifoam M-10

The basic antifoam preparation used in these experiments was 'Antifoam M-10' (Dow Corning Ltd., Barry, Glamorgan, U.K.). This had the following composition in g:

Polydimethylsiloxane (PDS)	9.5
Silica	0.5
Polyoxyethylene sorbitan monostearate (PSMS)	3.0
Glycerol monostearate (GMS)	2.0

Sodium carboxy methyl cellulose (SCMC)	1.5
Sorbic acid	0.075
Water	83.425

Other Antifoam Preparations

The antifoam preparations used to study the effect of constituents other than PDS in Antifoam M-10 on PDS binding by yeast were prepared in the laboratory. In the preparation of these antifoams, Antifoam M (Dow Corning Ltd.) which was a dispersion of silica (5.0%, w/w) in polydimethylsiloxane was used. The composition of these was the same as Antifoam M-10 except that the concentration of one constituent was varied.

BINDING OF POLYDIMETHYLSILOXANE (PDS) TO *SACCHAROMYCES CEREVISIAE*

Binding of PDS by *Saccharomyces cerevisiae*

Washed cells of *Saccharomyces cerevisiae* were suspended in 0.1M-KH_2PO_4 buffer (pH 4.5) to a concentration of 2.5 mg dry weight equivalent per ml. Portions of this suspension (40 ml) were pipetted into 250 ml conical flasks and to each flask a 0.1% (w/v) solution of antifoam emulsion (10 ml) in 0.1M-KH_2PO_4 buffer (pH 4.5) was added. The flasks were incubated at 30°C on an orbital shaker (G-10 Gyrotory Shaker, New Brunswick Scientific Co. Inc.,New Brunswick, U.S.A.) at 300 rev per min. Samples were removed at predetermined time intervals, and the organisms were removed by centrifugation at about 1000 g. The supernatant was removed carefully and the PDS content of the yeast pellet was then estimated.

Extraction and Estimation of PDS

The yeast pellet was suspended in water to a concentration of approximately 4.0 mg dry weight equivalent per ml and transferred into a 100 ml screw-capped glass bottle. 4-Methyl-pentan-2-one (0.5 ml per ml of suspension) was then added to the bottle and shaken on a Griffin flask shaker (Griffin and George Ltd., Middlesex,U.K.) for 30 min. The contents of the bottle was centrifuged for one minute and top MIBK (methyl isobutyl ketone) layer was removed carefully with a Pasteur pipette. The amount of PDS in the sample was estimated using a Pye-Unicam SP 90A series 2 atomic absorption spectrophotometer (Pye-Unicam Ltd.,Cambridge U.K.). A blank of MIBK was used to zero the instrument. Silica present in the yeast pellet does not interfere with the estimation of PDS by this method (Neal, 1969).

ENZYMIC AND CHEMICAL TREATMENT OF *SACCHAROMYCES CEREVISIAE*

Enzymic and Potassium Hydroxide Treatments

Washed organisms (100 mg dry weight equivalent) were suspended in a solution (10 ml) of trypsin, β(1-3)-glucanase or potassium hydroxide. The suspension was incubated on an orbital shaker (at 300 rev per min) at 30°C. Details of the conditions for the treatments are given in Table 1.

After treatment, the organisms other than those treated with β(1-3) glucanase were washed three times in 0.1M KH_2PO_4 buffer (pH 4.5). The washed organisms were suspended in 40 ml of the same buffer and used as a sample to study PDS binding. Organisms

Reagent	Buffer	Concentration of the reagent	pH
Trypsin	0.05M Tris-HCl	0.5 mg per ml	9.0
β(1-3) glucanase	1.0M sorbitol containing (per litre) Imidazole 0.68 g, $MgCl_2$ 2.03 g	5 units[a] per mg dry / wt equiv of yeast	6.0
Potassium hydroxide	-	60.0 mg per ml	-

Table 1. Conditions used for treating *Saccharomyces cerevisiae*.

a One unit of β(1-3) glucanase is the amount which liberates one mole glucose per min from laminarin substrate (Alterthum & Rose, 1973).

treated with β(1-3)glucanase were washed in 1.0M sorbitol (pH 4.5). The washed organisms were suspended in 40 ml of the same solution and used as a sample to study PDS binding.

The methods used to treat intact cells of *Saccharomyces cerevisiae* with trypsin and potassium hydroxide were modifications of the methods used by Lyons & Hough (1970b, 1971) and McMurrough & Rose (1967), respectively, to treat cell walls of yeast with the same reagents.

Hydrofluoric Acid Treatment

Freeze-dried organisms (100 mg) were incubated with hydrofluoric acid (58 - 62%, v/v; 1.0 ml) at 0°C in a polythene tube for 3 - 5h. The tube was then cooled to about -60°C in a bath of solid CO_2/ethanol and a calculated quantity of 2N KOH was then added almost to neutralize the hydrofluoric acid. The mixture was then allowed to warm to 0°C and the pH value adjusted to 7.0 with solid K_2CO_3. The organisms were then centrifuged and washed five times with distilled water. The washed organisms were freeze-dried and stored at 4°C over silica-gel in a vacuum desiccator. Treatment of isolated cell walls, with the above mentioned reagents, was carried out by using methods identical to those used to treat whole cells.

PREPARATION OF YEAST CELL WALLS

In the preparation of yeast cell walls, the method of McMurrough & Rose (1967) was used to separate cell walls from intact organisms.

Washed yeast cells (200 - 300 mg dry weight equivalent) in 20 ml of 0.1 M KH_2PO_4 buffer (pH 4.5) were shaken at 0°C, in a homogeniser (B. Braun apparatebau, Melsungen, W. Germany) with 30 g of glass beads (Glasperlen, 0.17 - 0.18 mm diameter, B. Braun apperatebau) for 2 min at 4000 rev/min. The homogeniser bottle was cooled with a spray of liquid carbon dioxide. The beads were allowed to separate and the suspension of unbroken cells and cell walls was decanted. The beads were washed a few times with ice-cold 0.1M KH_2PO_4 buffer and the washings were pooled with the suspension of unbroken cells and cell walls. The suspension was then centrifuged at 1300 g for 15 min at 0°C. The mixture of unbroken cells and cell walls was washed five times in ice-cold KH_2PO_4 buffer and the upper layer of the pellet, which contains cell walls, was separated by suspending in distilled water, care being taken not to disturb the lower layer of unbroken cells. The walls were washed ten times in ice-cold distilled water. Absence of intact organisms from the cell-wall preparation was established by microscopic examination of methylene blue-stained preparations (Chattaway, 1968). The walls were then freeze-dried and stored at 4°C over silica gel in a vacuum desiccator.

ANALYTICAL METHODS

Determination of Carbohydrate

The glucan and mannan content of cell walls of *Saccharomyces cerevisiae* were determined by the method of McMurrough & Rose (1967) which is a modification of the sulphuric acid/carbazole differential

extinction method originally described by Dische (1927, 1930) and later embellished by Gurin & Hood (1939),Siebert & Atno (1946) and Holzman, MacAllister & Niemann (1947).

Duplicate portions (0.5 ml) of suspensions containing the equivalent of 100 - 500 µg dry weight of cell walls were dispensed into boiling tubes and aqueous sulphuric acid (5.0 ml; 8 vol. conc. H_2SO_4: one vol. of water) was added to each tube. The tubes were then vigorously shaken and allowed to cool at room temperature. Carbazole reagent (0.3 ml, containing 0.5%, w/v, carbazole in 95% v/v ethanol) was added to each tube. The tubes were covered with aluminium caps, the contents rapidly mixed, and tubes placed in a bath of boiling water for 10 min. After cooling the extinctions of the solutions were measured at 435 nm and 535 nm in cuvettes (one cm path length) in a Unicam SP 500 spectrophotometer. Standard solutions of glucose, mannose and glucose with mannose were assayed in triplicate with each determination. The ratio of glucose to mannose in samples of hydrolysed cell walls were estimated from values obtained for the ratio E_{535}/E_{435} with calibration curves in which values for this ratio, obtained by using mixtures of known composition, were plotted against the percentage of glucose and mannose in the mixtures. Then from the E_{535} reading, the absolute amounts of each hexose could be estimated.

Determination of Protein

The total nitrogen content of yeast walls was determined by the micro-Kjeldahl technique.

A portion of suspension (0.5 ml) containing about 6 mg dry weight per ml was placed in a micro-Kjeldahl flask and 0.5 ml of the digestion mixture (conc. H_2SO_4 containing three Kjeldahl catalyst tablets, selenium, per 100 ml; B.D.H. chemicals Ltd., Poole, Dorset, U.K.) was added. The mixture was then refluxed in a micro-Kjeldahl apparatus for about one hour until it became colourless. Then the mixture was neutralized with saturated NaOH and made up to 10 ml with ammonia-free distilled water.

A colorimetric method described by Searcy, Reardon & Foreman (1967) and Fraser & Russel (1969) was used to determine the nitrogen content of the micro-Kjeldahl digest.

To 0.5 ml of this digest 1.0 ml of reagent A and 1.0 ml of reagent B were added, mixed and incubated in a water bath at 37°C for 10 min. Reagent A contained 8.5% (w/v) sodium salicylate and 0.06% (w/v) sodium nitroprusside in ammonia-free distilled water. Reagent B contained 0.25% (w/v) sodium dichloroisocyanurate in 0.3N NaOH. The mixture was then made up to five ml with ammonia-free distilled water and mixed thoroughly. The extinction of this solution at 660 nm was measured in a Unicam SP 600 spectrophotometer against a reagent blank, using cuvettes of one cm path length. The protein content was calculated by multiplying the value for total nitrogen by a factor 6.3.

Determination of Phosphorus

For the determination of phosphorus in yeast walls the method of Chen, Toribara and Warner (1956) which is a modification of the original methods described by Ammon & Hinsberg (1936) and Lowry *et al.*

(1954) was used. Yeast walls (about 10 mg dry weight) were weighed into a Pyrex glass tube and five drops of the ashing acid mixture (perchloric, 72%, v/v : conc sulphuric, 3:2) were added . The tube was then heated on an electric digestion rack in a fume cupboard until the liquid became clear. The mixture was then cooled and made up to 25 ml with distilled water. One ml of this solution was made up to 4.0 ml with distilled water and 4.0 ml of a reagent mixture containing one volume of 6N sulphuric acid, two volumes of distilled water, one volume of ammonium molybdate (2.5%, w/v) and one volume of ascorbic acid (10%, w/v) were added. The solution was thoroughly mixed and incubated in a water bath at 37°C for 1 - 2 h. The extinction of this solution at 820 nm was measured using a Unicam SP 600 spectrophotometer against a reagent blank and cuvettes of one cm path length.

MEASUREMENT OF ELECTROPHORETIC MOBILITY OF *SACCHAROMYCES CEREVISIAE*

The electrophoretic mobility of yeast at different pH values was measured using a Carl Zeiss Cytopherometer (Carl Zeiss Oberkochen, W. Germany) by a modification of the method by Somers & Fisher (1967). Movement of cells was timed over approximately 80 µm in both directions (current reversal). Each mobility value was the mean of at least 20 observations. Mobility was measured at 30°C using yeast suspensions, in the appropriate buffer, containing 0.5 mg dry weight equivalent or 10^7 organisms per ml. The buffers used in these experiments (Gittens & James, 1963) were as follows:

pH 1.5 and 2.0	NaCl/HCl
pH 3.0 - 9.0	NaCl/sodium acetate/sodium barbiturate/HCl
pH 10	NaCl/sodium acetate/sodium barbiturate/NaOH

Buffers had an ionic strength of 0.05 or 0.005. The conductivity of buffers was measured with a Griffin conductivity bridge (Griffin & George Ltd.,Middlesex, U.K.).

STAINING OF *SACCHAROMYCES CEREVISIAE* WITH FLUORESCENT ANTIBODY.

Growth of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae NCYC 366 was grown in Saboraud dextrose broth for three days at 30°C with stirring by means of the apparatus described by Patching & Rose (1970). The medium had the following composition in g per litre of distilled water:

Mycological peptone	10.0
Glucose	40.0

The medium was sterilized by autoclaving at 15 lb/in^2 (10.34 x 10^4 pa) for 15 min.

The cells were harvested by centrifuging at 2000 g and washed three times in sterile 0.1M phosphate buffer (pH 7.0). The washed cells were suspended to a concentration of approximately 10^8 cells per ml in sterile 0.85% (w/v) saline and stored in sterile screw-capped bottles at -20°C. All operations were carried out under sterile conditions.

Production of Antiserum

Anti-yeast antiserum was prepared by the method described by Klaushofer & Dorfwirth (1970). Healthy adult rabbits (approximately

2.5 - 3.0 kg weight) were injected with the preparation of *Saccharomyces cerevisiae* NCYC 366, using the following routine.

The figures below refer to the volume (ml) of the yeast suspension injected into the peripheral vein of the ear.

Day 1	1.0
Day 2	1.2
Day 8	1.6
Day 12	2.5
Day 16	1.3
Day 20	2.5
Day 24	2.5

On the 28th day, approximately 30 ml of blood was taken from the ear vein, allowed to clot and the serum separated after standing at $4^{o}C$ overnight. The titre of the serum was estimated by immunofluorescent staining. The serum was stored under liquid nitrogen in sealed ampules.

Immunofluorescent Staining Procedure

The immunofluorescent staining of yeast was carried out by the method described by Richards & Cowland (1967).

The yeast cells were washed several times in distilled water and the concentration of the suspension adjusted to approximately 1.5×10^{8} cells per ml. A smear was made on a grease-free microscope slide, air-dried and fixed in acetone for 10 min. The smear was covered with inactive calf serum (Wellcome Reagents Ltd., Beckenham, Kent, U.K.) for 15 min, followed by washing in saline (0.85%, w/v) buffered to pH 7.2 with phosphate buffer (0.01M;

Cowland, 1968) for 30 min. The specific (rabbit) anti-yeast serum was then added to the slide at its predetermined titre and allowed to react for 30 min, after which time the serum was removed by washing in phosphate-buffered saline for another 30 min. Finally the area of the smear was covered with anti-rabbit (sheep) immunoglobin (fluorescein labelled ; Wellcome Reagents Ltd.) for another 30 min, followed by a final wash of 40 min in phosphate-buffered saline. The smear was then mounted in phosphate-buffered glycerol (10%, w/v, pH 7.2, Richards & Cowland, 1967) and the coverslip was sealed with paraffin.

Microscopic Examination of Smears

Microscopic examination of smears was carried out using a Leitz Wetzler 'ORTHOPLAN' microscope (Ernst Leitz GMBH, Wetzler, Germany) fitted with a Leitz Wetzler fluorescein vertical illuminator having a filter combination recommended for fluorescein isothiocyanate microscopy. The smears were illuminated with ultraviolet radiation, the source being an Osram H.B. 200 mercury vapour lamp.

Ultraviolet Photography

Photographic records were made with Kodak Ektachrome ER film using a Leitz Wetzler 'ORTHOMAT' camera fitted to the microscope. The film speed used was 400 ASA (27 DIN).

STAINING OF *SACCHAROMYCES CEREVISIAE* WITH FLUORESCEIN ISOTHIOCYANATE-CONJUGATED CONCANAVALIN A

Conjugation of Fluorescein Isothiocyanate to Concanavalin A.

Fluorescein isothiocyanate was conjugated to concanavalin A by the method of Tkacz *et al.* (1971).

Fluorescein isothiocyanate was dissolved (0.94 mg per ml) in 0.1M Na_2HPO_4. To 0.48 ml of this solution was added 11.9 mg of concanavalin A in 0.6 ml of 1.0M NaCl. The reaction mixture was incubated at room temperature (ca. 22°C) for 3 h and then dialyzed overnight at 4°C against 2 litres of 1.0M NaCl. After removing any precipitate by centrifugation at 0°C the mixture was purified using a column of Sephadex G-75. This product was further purified by dialysing against 2 litres of 1.0M NaCl at 4°C overnight. The purified product was stored at -20°C in sealed ampules.

Staining of *Saccharomyces cerevisiae* with Fluorescein Isothiocyanate-Concanavalin A conjugate

The method of staining used was as described by Tkacz *et al.* (1971). However the mounting solution used was phosphate-buffered glycerol (10%, w/v; pH 7.2) described by Richards & Cowland (1967).

Ultraviolet Microscopy and Photography

The methods used for these studies were the same as those described for immunofluorescence studies of yeast.

MEASUREMENT OF INVERTASE ACTIVITY OF *SACCHAROMYCES CEREVISIAE*

Invertase (β-D-fructofuranosidase, fructohydrolase, E.C. 2.2.1.26) was assayed by a method based on that of Sutton & Lampen (1962). The reaction mixture was as follows:

Nystatin suspension (200 µg/ml)	0.25 ml
Cell suspension (200 µg dry weight equivalent/ml)	0.25 ml
Sucrose solution (10%, w/v) in acetate buffer (pH 5.0)	0.50 ml

The solutions were mixed thoroughly and incubated in a water bath at 30°C for 15 min. The reaction was stopped by adding a spatula-tip full of $CaCO_3$ powder and heating in a bath of boiling water for 5 min. It was necessary to add $CaCO_3$ to prevent acid hydrolysis of sucrose. After decanting the supernatant and diluting suitably, the glucose present was assayed using a blood sugar (GOD-perid method) test kit (Boehringer GMBH-Mannheim, W. Germany). This test kit was used according to the instructions supplied, except that deproteinisation was found to be unnecessary. The invertase activity was calculated directly, after subtraction of the control value. The control value was obtained by boiling a test sample with calcium carbonate for five minutes before incubating with sucrose.

EXTRACTION OF LIPIDS FROM *SACCHAROMYCES CEREVISIAE*

Lipids were extracted from *Saccharomyces cerevisiae* by the method of Letters (1968) with the modification by Hunter & Rose (1972). Freeze-dried cells of *Saccharomyces cerevisiae* (200 mg)

were suspended in hot ethyl alcohol (20 ml, 95%, v/v) and maintained at 80°C for 15 min, with stirring. Then the suspension was cooled to room temperature and filtered. The cells were resuspended in methyl alcohol (15 ml) and stirred at room temperature for 10 min. To this chloroform (15 ml) was added and stirred for 3 h at room temperature. The cells were filtered and the methyl alcohol/chloroform treatment was repeated twice more for 3 h and 2 h respectively. Then the cells were filtered washed four times in distilled water and freeze-dried.

MEASUREMENT OF FLOCCULATION OF *SACCHAROMYCES CEREVISIAE*

Flocculation of *Saccharomyces cerevisiae* was measured by a modification of the methods described by Mill (1964a) and Greenshields *et al.* (1972). Yeast cells were suspended to a concentration of approximately 4.0 mg dry weight equivalent per ml in either 0.05M sodium acetate buffer (pH 4.5) containing 0.1% (w/v) calcium chloride or in deionized water. A portion (3 ml) of this suspension was transferred into a cuvette (1 cm path length) and the extinction at 600 nm was measured against a blank in a Unicam SP 1800 spectrophotometer (Pye-Unicam Ltd.) with an automatic pen-recorder over a period of 5 - 10 min. Extinction values were converted to mg dry weight equivalent per ml using a calibration curve and a graph plotted of dry weight of yeast per ml against time. Sedimentation rates (S.R.) were calculated by measuring the maximum slope on the curve and are expressed

as µg dry weight per ml per min.

MEASUREMENT OF CALCIUM BINDING BY WALLS OF *SACCHAROMYCES CEREVISIAE*

Calcium binding by isolated cell walls was measured by suspending freeze-dried walls (50 mg dry weight), that had been washed in 10 mM EDTA, in 0.05M sodium acetate buffer (pH 4.5, 10 ml) containing 3.6 mmol $CaCl_2$ and approximately 0.4mCi of $Ca^{45}Cl_2$ per litre (Helm *et al.*, 1953). The reaction mixture was placed in a 50 ml conical flask containing a polythene-covered magnet (2 cm long). The flask was incubated in a water bath maintained at 30°C. The walls were kept suspended by stirring with a magnetic stirrer. Samples (2 ml) were withdrawn after 40 min and filtered through membrane filters (2.5 cm diam, 0.45 µm pore size; Oxoid Ltd., London U.K.) that has been washed with 10 ml portions of 3.6 mM $CaCl_2$ in sodium acetate buffer (pH 4.5). After filtration the cells and filters were immediately washed with 10 ml portions of the same buffer solution. Using forceps the filters were transferred into scintillation vials (20 ml capacity, Beckman Instrument Ltd., Glenrothes, Fife, Scotland) containing 10 ml of Unisolve scintillation fluid (Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K.). The radioactivity of the samples was measured using a 'Phillips Liquid scintillation Analyser'. The amount of calcium bound is expressed as µg per 100mg dry weight of walls.

MATERIALS

Chemicals

All chemicals other than those listed below or stated otherwise were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K., and were of 'Analar' grade when available.

Supplier	Chemicals
1. Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K.	1. Polyoxyethylene sorbitan monostearate (Tween 60)
2. Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, U.K.	1. 4-methyl pentan-2-one 2. Hydrofluoric acid (58 - 62% v/v)
3. Pharmacia (G.B.) Ltd., London, U.K.	1. Concanavalin A
4. Calbiochem Ltd., London, U.K.	1. Fluorescein isothiocyanate
5. Sigma London Chemical Co. Ltd., Surrey, U.K.	1. Sodium dichloroisocyanurate

Enzymes

Crystalline trypsin was obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K. and a preparation of β(1-3) glucanase was

supplied by Dr. T.G. Cartledge of the School of Biological Sciences, University of Bath. This β(1-3) glucanase was obtained from the culture filtrate of basidiomycete QM 806 by the method of Alterthum & Rose (1973).

Antibody Preparations

Fluorescein labelled anti-rabbit (sheep) immunoglobulin was obtained from Wellcome Reagents Ltd., Beckenham, U.K.

Culture Media Ingredients

All ingredients for the preparation of culture media were supplied by Oxoid Ltd., London, U.K. unless otherwise stated.

RESULTS

PART I

POLYDIMETHYLSILOXANE (PDS) BINDING BY *SACCHAROMYCES CEREVISIAE*

TIME-COURSE OF PDS BINDING BY *SACCHAROMYCES CEREVISIAE* NCYC 366

The kinetics of PDS binding by *Saccharomyces cerevisiae* have been studied in detail by Vernon & Rose (1976). The time-course of PDS binding by *Saccharomyces cerevisiae* NCYC 366 from 0.1M KH_2PO_4 buffer (pH 4.5) containing Antifoam M-10 was re-investigated as this was to be used as the control system in the present study. The method and the strain of *Saccharomyces cerevisiae* used in this experiment were the same as those used by Vernon & Rose (1976).

It was found that *Saccharomyces cerevisiae* NCYC 366, that had been grown in defined medium and harvested in the mid-exponential phase of growth, when suspended at 2.0 mg dry weight equivalent per ml in 0.1M KH_2PO_4 buffer (pH 4.5) containing Antifoam M-10 (200 µg emulsion per ml) became saturated with PDS after incubation for 3 h at 30°C. The saturation concentration of PDS bound by the organisms was approximately 2.1 µg per mg dry weight equivalent (2×10^7 organisms) of yeast (Fig. 8). The results of this experiment were found to be reproducible with good accuracy when antifoam emulsions of same chemical composition and physical nature were used. However antifoam emulsions even with the same chemical composition but having different droplet size gave non-reproducible results when used in this experiment. Therefore the droplet size of the emulsions was examined under the microscope before use and only emulsions with comparable droplet sizes were used in this experiment.

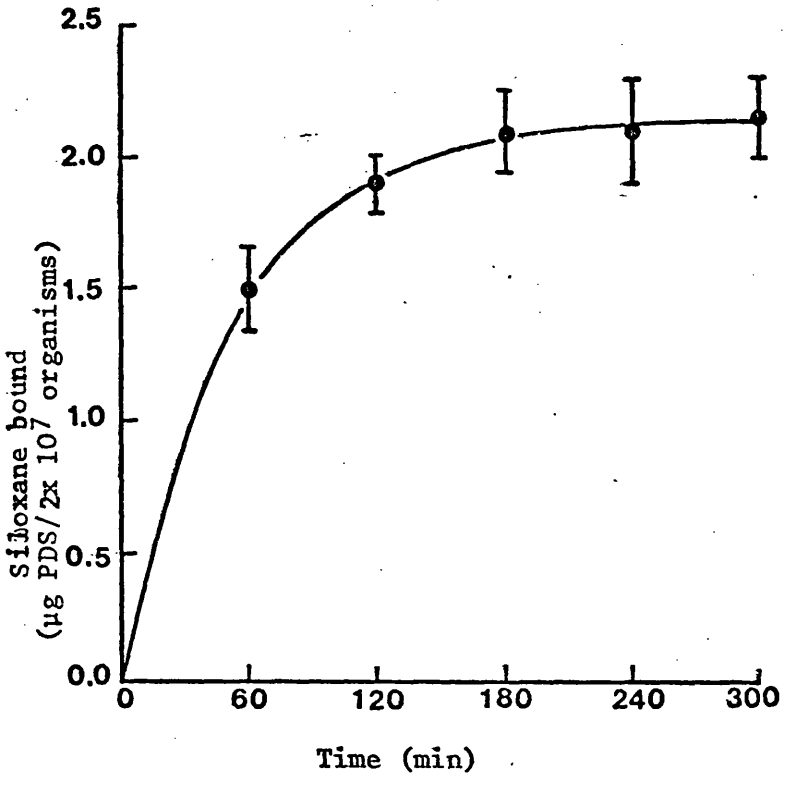


Figure 8 Time-course of PDS binding by *Saccharomyces cerevisiae* NCYC 366 from 0.1M KH_2PO_4 (pH 4.5) containing 200 µg Antifoam M-10 emulsion per ml. Organisms were grown in defined medium and harvested in the mid-exponential phase of growth. Values indicated are the means of four determinations. Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

EFFECT OF INCLUDING INGREDIENTS OTHER THAN SILOXANE IN THE ANTIFOAM EMULSION ON THE SATURATION CONCENTRATION OF PDS BOUND BY *SACCHAROMYCES CEREVISIAE* NCYC 366.

The effect of the eumulsifiers, polyoxyethylene sorbitan monostearate (PSMS) and glycerol monostearate (GMS) and the thickner, sodium carboxymethyl cellulose (SCMC) on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366 was studied by varying the concentration of these ingredients in the antifoam emulsion. The concentration of each ingredient was varied while the concentration of all other components of Antifoam M-10 were kept constant. It was not possible to prepare a stable emulsion without any PSMS.

The antifoam emulsions were prepared by using Antifoam M (Dow Corning Ltd., Barry, U.K.) which is a dispersion of silica (5.0%, w/w) in PDS. The weighed ingredients were melted by warming in a 250 ml conical flask and the molten ingredients were thoroughly mixed in a blender (Oesterizer, Rima Electrical Ltd., Middx., U.K.) at maximum speed for one minute. The resulting mixture was passed several times through a bench homogeniser (Ormerod Engineers Ltd., Homogeniser QP Type DF 1 LP, H.P. 0.06, working Pressure 6.9×10^5 Pa) until desired emulsification was achieved. The emulsions were examined microscopically for homogenity in droplet size. Only emulsions with droplet size measuring about 10 - 20 μm diam were used in these experiments.

The effect of emulsifier concentration in the antifoam emulsion on the saturation concentration was studied by suspending yeast cells at 2.0 mg dry weight equivalent per ml in 0.1M KH_2PO_4 buffer (pH 4.5) containing 200 µg antifoam emulsion per ml. The saturation concentration was determined after incubation for 4 h at 30°C. A control experiment using antifoam M-10 was run parallel with each experiment. It was observed that, irrespective of the concentration of the emulsifier or thickner in the antifoam emulsion, saturation concentration was reached after 3 - 4 h incubation at 30°C.

The saturation concentration varied with the concentration of emulsifier in the antifoam. Lowering the concentration of any one of the two emulsifiers caused an increase in the saturation concentration in a linear manner (Figs. 9 and 10).

The saturation concentration was also related to the Hydrophilic-Lipophilic-Balance (HLB) of the corresponding antifoam emulsion. The HLB values were calculated for each emulsion using the following equation (Shaw, 1970).

$$HLB = (x \times p/100) \quad (y \times q/100)$$

where x and y are the respective HLB values of the emulsifiers and p and q are the respective percentages (w/w) of the emulsifiers in the antifcam emulsion.

The calculated HLB values for antifoam emulsions with different emulsifier combinations are given in Table 2. The HLB value indicate the relative stability of the emulsion, and the optimum

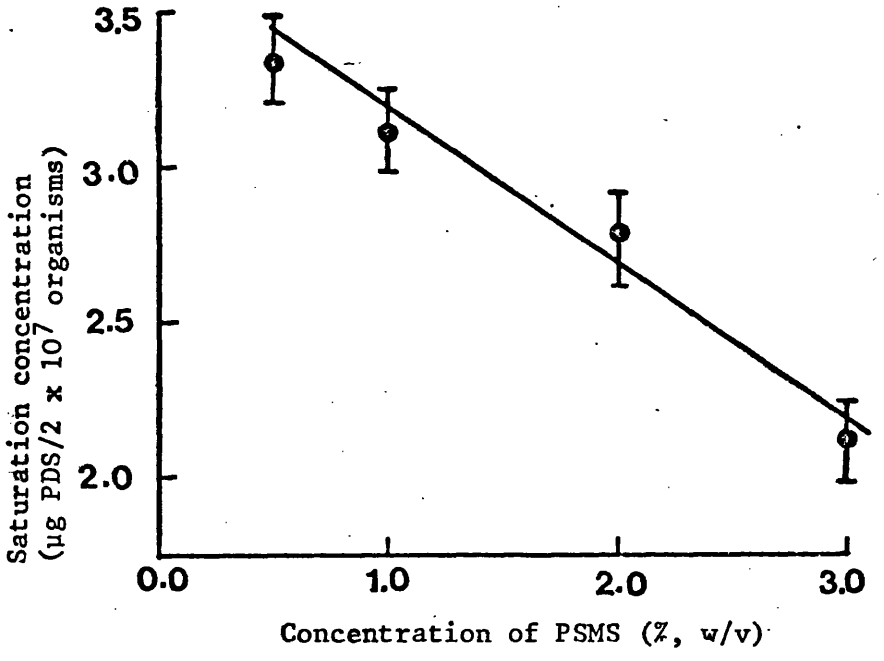


Figure 9 Effect of concnetration of PSMS in the antifoam emulsion on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366, that had been grown in defined medium and harvested in the mid-exponential phase of growth.

Values indicated are the means of four determinations. Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

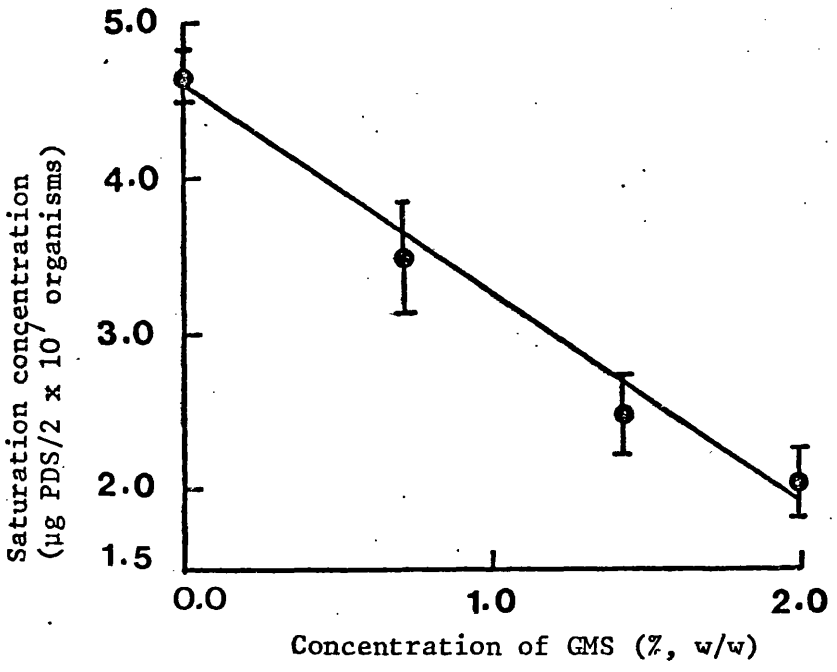


Figure 10 Effect of concentration of GMS in the antifoam emulsion on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366, that had been grown in defined medium and harvested in the mid-exponential phase of growth.

Values indicated are the means of four determinations. Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

Concentration (Percent w/w)		HLB
PSMS	GMS	Value
3.0	0.0	14.9
3.0	0.7	12.6
3.0	1.4	11.3
3.0	2.0	10.4[a]
2.0	2.0	9.3
1.0	2.0	7.4
0.5	2.0	5.9

Table 2 HLB values of the antifoam emulsions containing different emulsifier combinations. a indicates the value for Antifoam M-10 which is the standard antifoam used in this study.

HLB for oil-in-water emulsions into which category silicone emulsions fall is in the range 11 to 12 (Boyde, Parkinson & Sherman, 1972).

It was observed that a decrease in the concentration of PSMS in the antifoam emulsion caused a decrease in the HLB value whereas lowering the GMS concentration caused an increase. The saturation concentration of PDS bound by the yeast was found to increase with any deviation of the HLB value of the emulsions from 10.4, which is the HLB value for Antifoam M-10 (Fig. 11).

The effect of the concentration of SCMC on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366 was studied by employing the same method used to study the effect of emulsifiers. The concentration of SCMC in each antifoam emulsion was related to the saturation concentration of PDS bound by the yeast. The saturation concentration was found to decrease in a linear manner with the decrease of the concentration of SCMC (Fig. 12).

BINDING OF PDS BY *SACCHAROMYCES CEREVISIAE* NCYC 366 AFTER TREATMENT WITH DIFFERENT REAGENTS

There is a fair amount of evidence from the work of Vernon & Rose (1976) that it is the cell wall of *Saccharomyces cerevisiae* that is responsible for binding PDS. The cell wall

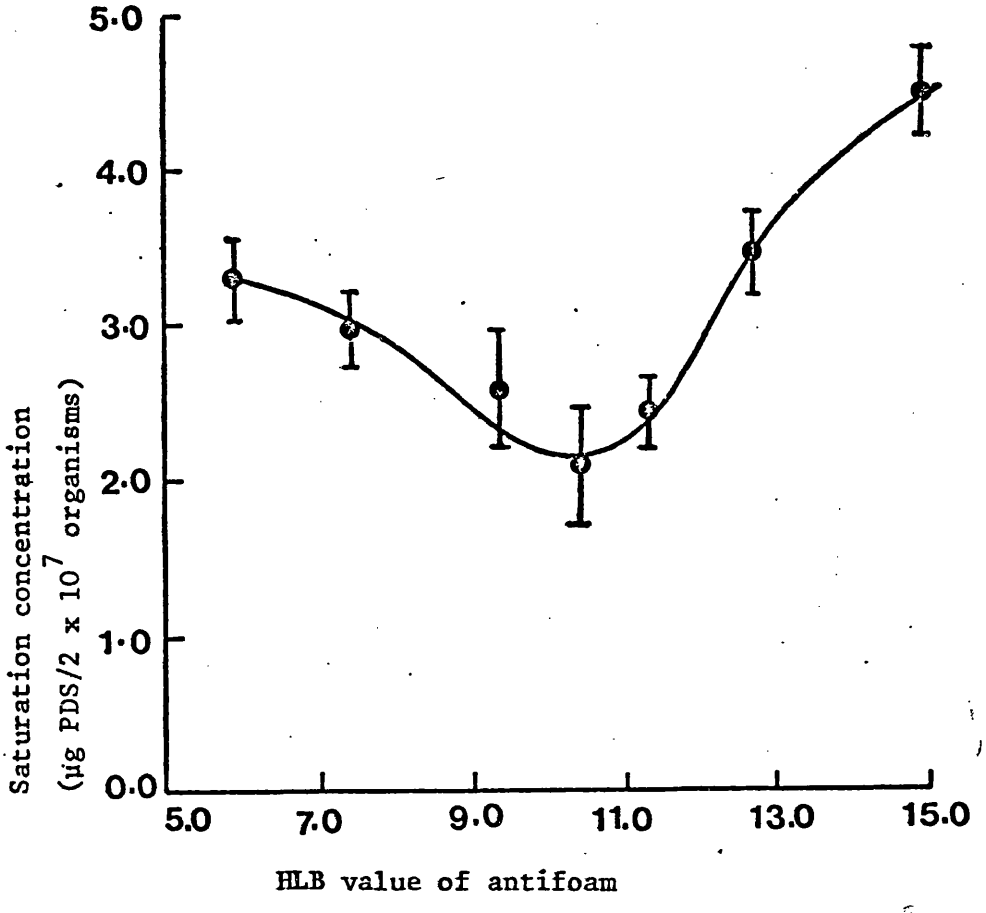


Figure 11 Effect of the Hydrophilic-Lipophilic-Balance (HLB) value of the antifoam on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366, that had been grown in defined medium and harvested in the mid-exponential phase of growth.

Values indicated are the means of four determinations Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

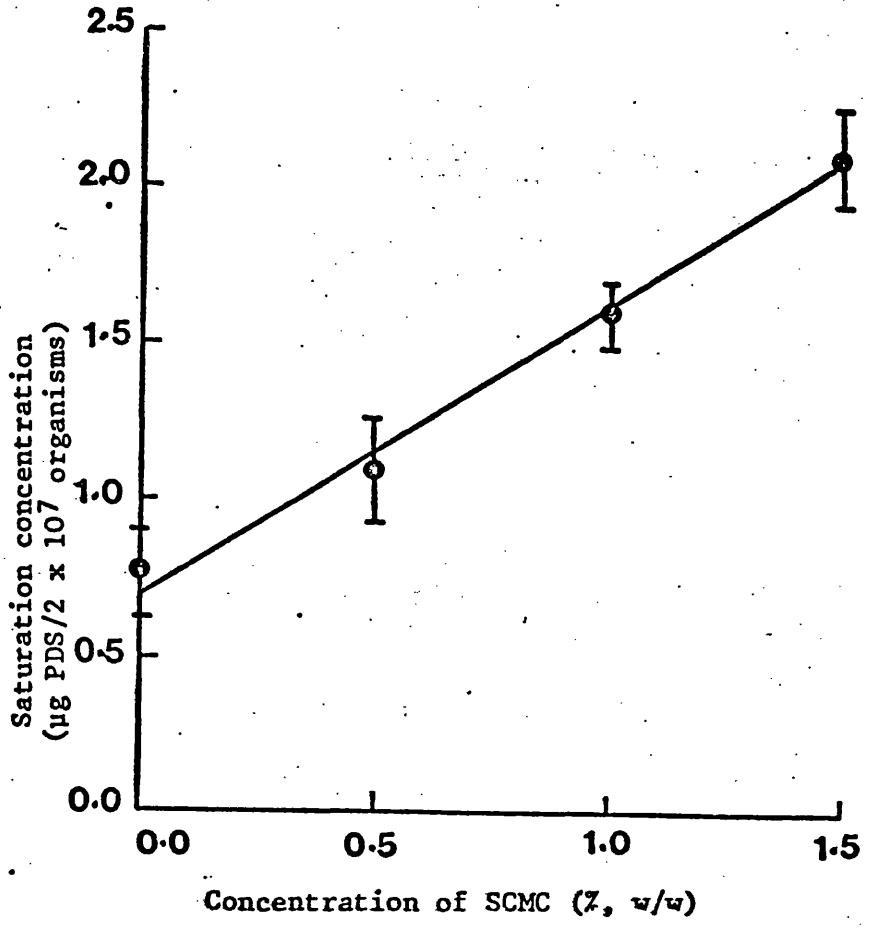


Figure 12 Effect of concentration of SCMC in the antifoam emulsion on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366 that had been grown in defined medium and harvested in the mid-exponential phase of growth.

Values indicated are the means of four determinations. Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

of *Saccharomyces cerevisiae* is a heterogeneous and highly complex organelle composed mainly of glucan, mannan, protein, N-acetyl glucosamine (some of which is present as chitin) and some lipid. Due to the complex nature of the cell wall it was necessary to investigate the nature of the components that are responsible for binding PDS. A possible method of doing so was to remove specific components of the cell wall of intact organisms by chemical and enzymic treatment and to study the effect on PDS binding.

The effect of several enzymic and chemical treatments, which are reported to remove specific wall components, on PDS binding by intact *Saccharomyces cerevisiae* NCYC 366 was studied. The enzymes used were trypsin and a β(1-3) glucanase while the chemicals used were potassium hydroxide (6%, w/v) and hydrofluoric acid (58 - 62%, v/v). Binding of PDS was studied by incubating organisms (4×10^7 organisms per ml) in 0.1M KH_2PO_4 buffer (pH 4.5) containing Antifoam M-10 (200 µg emulsion per ml) at 30°C except with organisms treated with β(1-3) glucanase. In the latter case binding was studied in 1.0M sorbitol (pH 4.5) instead of 0.1M KH_2PO_4 buffer. The method used to study PDS binding was the same as described for untreated organisms in the Methods section.

Binding of PDS by *Saccharomyces cerevisiae* NCYC 366 Treated with Trypsin

Trypsin is a protease with a fairly narrow specificity. It catalyses preferentially hydrolysis of peptide bonds involving carboxyl groups of the amino acids arginine and lysine. Treatment

of isolated walls of *Saccharomyces cerevisiae* with trypsin has been reported to dissolve 10 - 20% of the wall (Eddy, 1958), probably due to removal of a phosphomannan-protein complex (Lyons & Hough, 1970b, 1971). This phosphomannan-protein complex was claimed by Lyons & Hough (1970b, 1971) to be located at or near the cell-wall surface.

The saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366 treated with trypsin for 2, 4, 6 and 8 h as described in the Methods section was studied. Treated organisms became saturated more slowly than untreated organisms. These organisms were therefore incubated for longer periods of time than untreated organisms for saturation to be reached. The saturation concentration was found to decrease exponentially with time of trypsin treatment and reach a constant value after 6 h treatment. This value was about 1.1 µg PDS per 2×10^7 organisms which is about half of the amount bound by untreated organisms (Figure 13).

Binding of PDS by *Saccharomyces cerevisiae* NCYC 366 Treated with β(1-3) glucanase

As the name implies β(1-3) glucanase catalyses hydrolysis of β(1-3) linkages in glucans. The major component of *Saccharomyces cerevisiae* glucan is a highly branched insoluble polymer of glucose containing a very high proportion of β(1-3) linkages (Manners *et al.*, 1973a). Basidiomycete β(1-3) glucanase has been used in the preparation of sphaeroplasts of *Saccharomyces cerevisiae* NCYC 366 by Alterthum & Rose (1973).

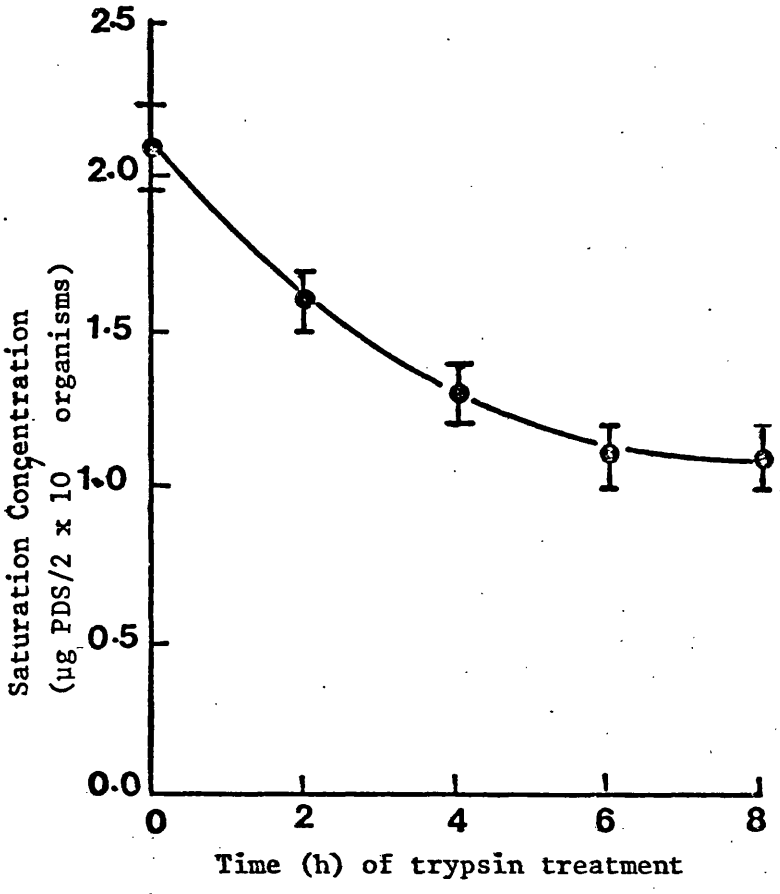


Figure 13 Effect of trypsin treatment on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366 that had been grown in defined medium and harvested in the mid-exponential phase of growth.

Values indicated are the means of four determinations.

Vertical bars indicate 95% confidence limits.

The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

Saccharomyces cerevisiae NCYC 366 after being subjected to β(1-3) glucanase treatment for 5, 10, and 15 min bound very large amounts of PDS in the range of about 4 - 6.5 μg PDS per 2 x 10^7 organisms. Organiams treated with β(1-3) glucanase bound PDS much faster and reached saturation more quickly than untreated organisms. However when the treatment was carried out for more than 15 min the saturation concentration of PDS bound by the organisms decreased to about 2.5 μg per 2 x 10^7 organisms (Figure 14).

Binding of PDS by *Saccharomyces cerevisiae* Treated with Potassium Hydroxide

Treatment of yeast cell walls with cold potassium hydroxide (6%, w/v) has been shown to extract most of the mannan, protein, soluble glucan and phosphorus (McMurrough & Rose, 1967). *Saccharomyces cerevisiae* NCYC 366 treated with potassium hydroxide (6%, w/v) bound PDS much slower than untreated organisms. Treated organisms became saturated only after incubation for longer periods of time than untreated organiams.. Organisms treated for 15 min were found to bind less than half the amount of PDS bound by untreated organisms. Longer periods of treatment only slightly altered the amount of PDS bound by the organisms (Figure 15).

Binding of PDS by *Saccharomyces cerevisiae* NCYC 366 Treated with Hydrofluoric Acid (58 - 62%, v/v).

Hydrofluoric acid has been successfully used as a reagent to

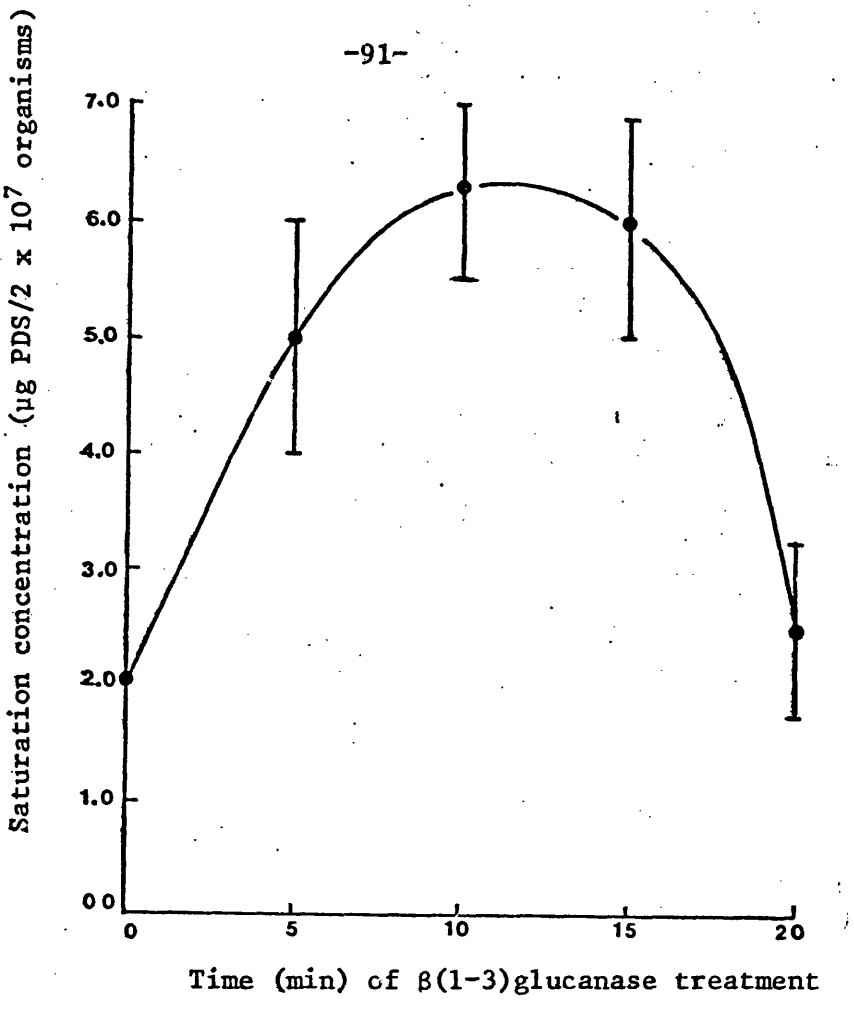


Figure 14 Effect of β(1-3) glucanase treatment on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366 that had been grown in defined medium and harvested in the mid-exponential phase of growth. Values indicated are the means of four determinations. Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

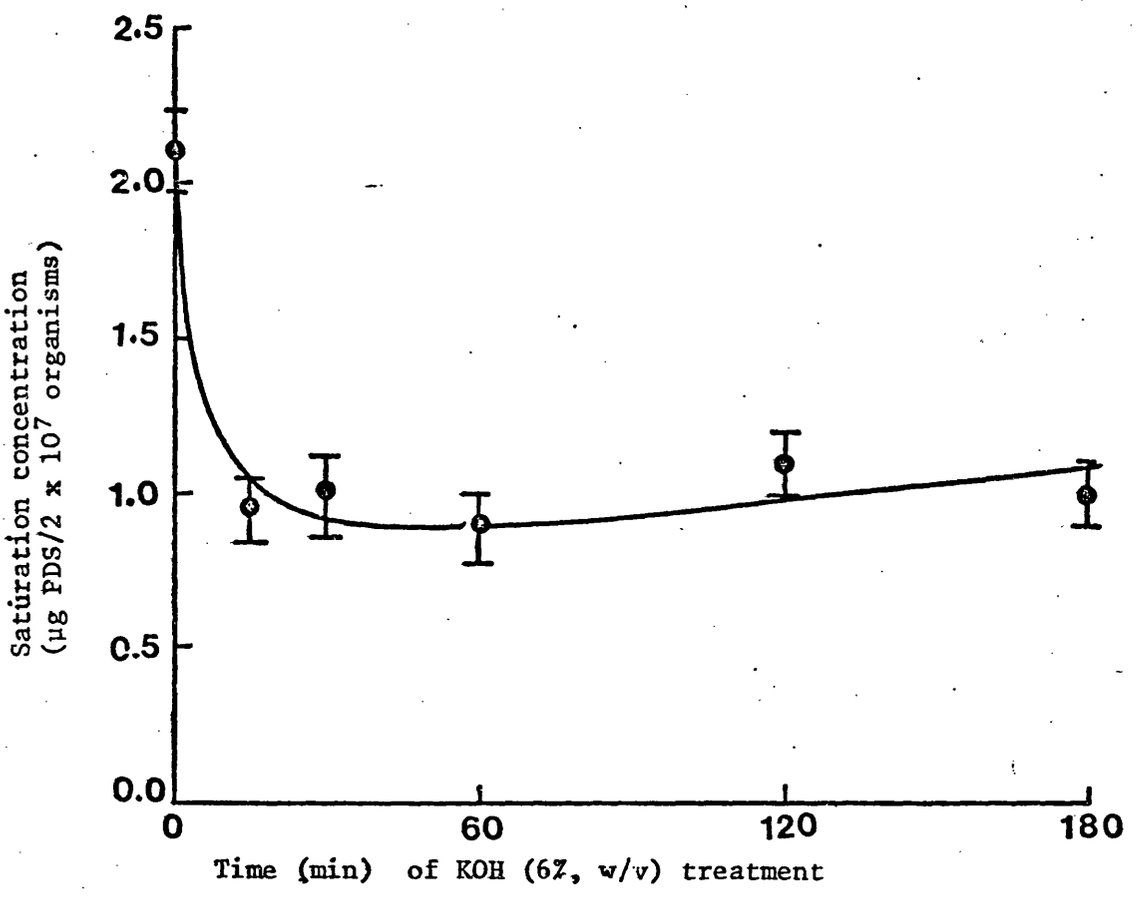


Figure 15 Effect of KOH (6%, w/v) treatment on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366 that had been grown in defined medium and harvested in the mid-exponential phase of growth.

Values indicated are the means of four determinations

Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7

cleave phosphodiester linkages in a variety of organic compounds. These compounds include teichoic acids (Burger & Glaser, 1964; Archibald, Baddiley & Shaukat, 1968), phosphomannan extracted from walls of *Saccharomyces cerevisiae* (Cawley *et al.*, 1972) and phospholipids extracted from bacteria (Shaw & Stead, 1974). The most interesting feature of the treatment with this reagent is that it does not cause any significant hydrolysis of glycosidic linkages. In view of these capabilities hydrofluoric acid (58 – 62%, v/v) was selected as one of the reagents for treatment of *Saccharomyces cerevisiae* NCYC 366 prior to studying PDS binding.

The time-course of PDS binding by *Saccharomyces cerevisiae* NCYC 366 treated with hydrofluoric acid (58 - 62%,v/v) for 3 - 5h at $0^{o}C$ was studied. The rate of uptake of PDS by hydrofluoric acid – treated organisms was slower than that of untreated organisms and the saturation concentration was about half of that of the control (Figure 16). Attempts to study the PDS binding by organisms treated for different periods of time as in previous experiments were not made due to hazards involved in handling concentrated hydrofluoric acid.

RELEASE OF PDS FROM *SACCHAROMYCES CEREVISIAE* NCYC 366 SATURATED WITH PDS BY TREATMENT WITH DIFFERENT REAGENTS

A study was made of the release of PDS from organisms that had been saturated with PDS, by treatment with trypsin, β(1-3) glucanase

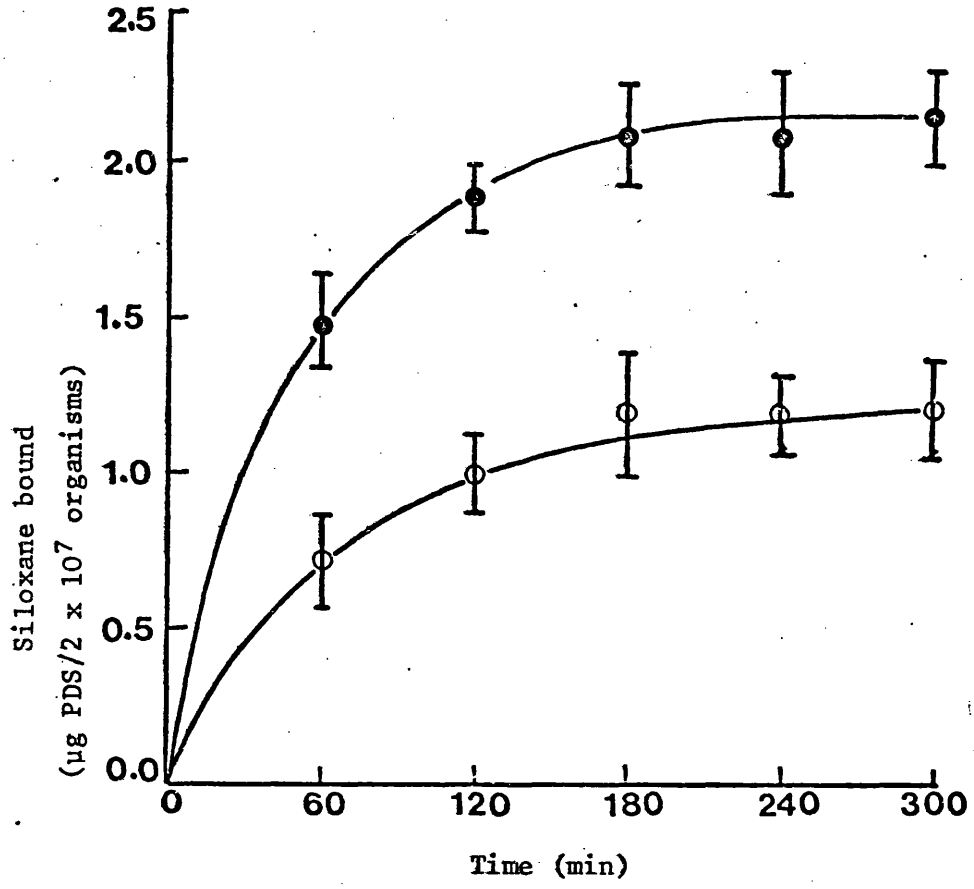


Figure 16 Time-course of PDS binding by *Saccharomyces cerevisiae* NCYC 366 after treatment with hydrofluoric acid (58 - 62%, v/v). ●——● indicates untreated organisms O——O hydrofluoric acid-treated organisms. The organisms were grown in defined medium and harvested in the mid-exponential phase of growth.

Values indicated are the means of four determinations. Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

or potassium hydroxide (6%, w/v). *Saccharomyces cerevisiae* NCYC 366 were saturated with PDS by suspending them at 2.0 mg dry weight equivalent per ml in 0.1M KH_2PO_4 buffer (pH 4.5) containing Antifoam M-10 (200 µg per ml) and incubating on an orbital shaker (300 rev per min) at 30°C for 4 h. The saturated organisms were treated with trypsin, β(1-3) glucanase or potassium hydroxide (6%, w/v) as described in the Methods section. After treatment for stated intervals of time organisms were washed once in distilled water and their PDS content estimated (Figures 17, 18 and 19).

Potassium hydroxide (6%, w/v) was the most effective in releasing PDS bound by these organisms. A maximum of about 85% of the PDS originally bound was released after treatment for 30 - 60 min. Trypsin treatment caused a release of about 81% of PDS originally bound after treatment for about 4 hours. Treatment with β(1-3) glucanase was less effective releasing only about 50 - 65% of the PDS originally bound (Table 3).

The PDS contents of organisms tended to drop and then increase slightly towards the end of the treatment probably due to re-adsorption of PDS from the suspension. Experiments on PDS release following hydrofluoric acid (58 - 62%, v/v) treatment were not carried out in view of the hazards involved.

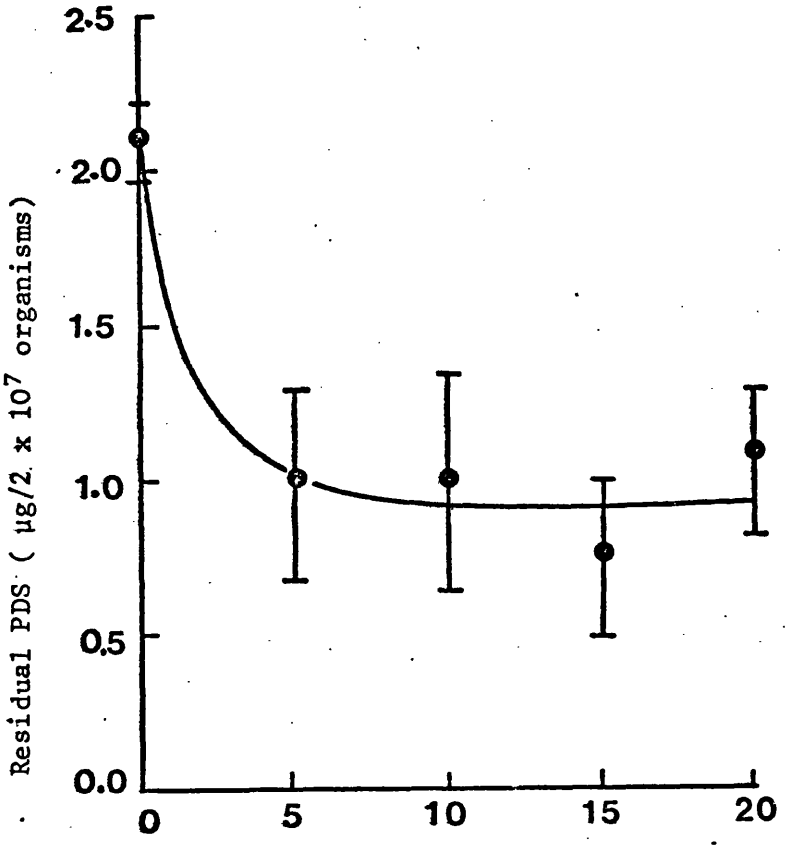


Figure 18 Effect of β(1-3) glucanase treatment on release of PDS from presaturated *Saccharomyces cerevisiae* NCYC 366 that had been grown in defined medium and harvested in the mid-exponential phase of growth.

Values indicated are the means of four determinations.

Vertical bars indicate 95% confidence limits.

The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

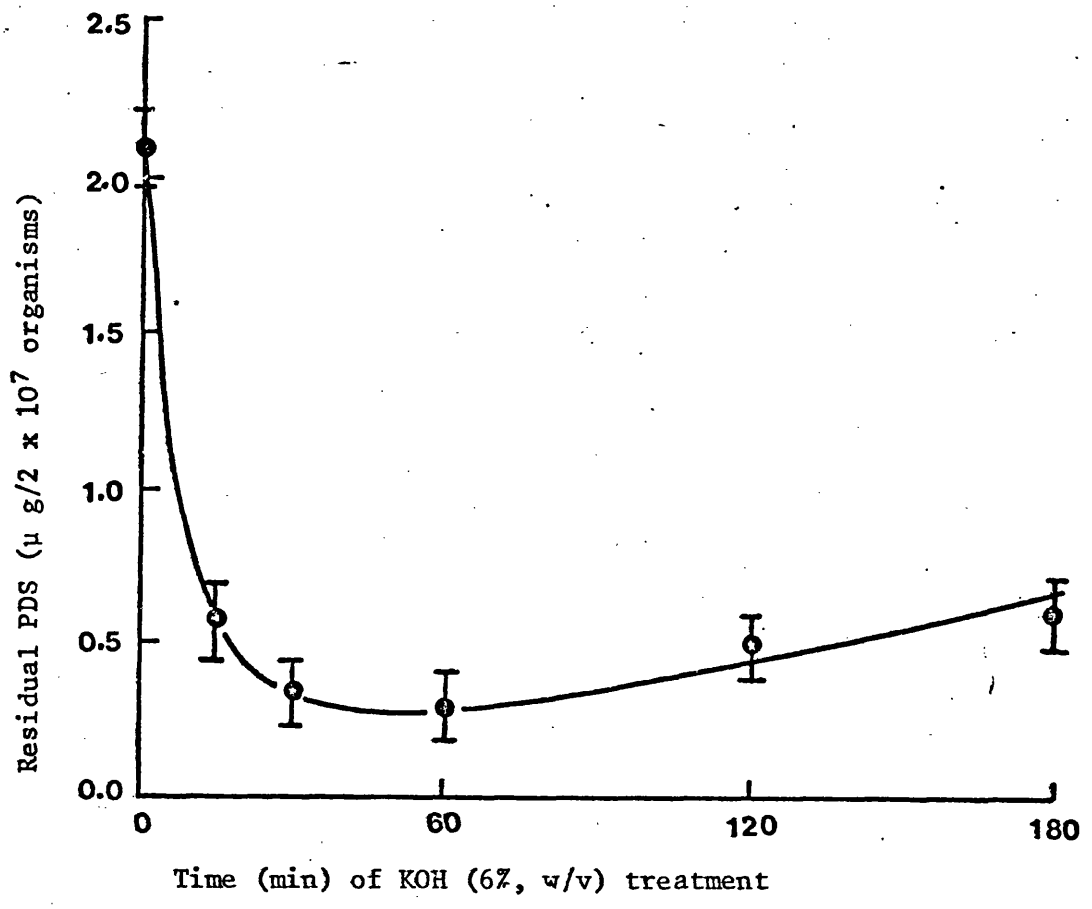


Figure 19 Effect of KOH (6%, w/v) treatment on release of PDS from presaturated *Saccharomyces cerevisiae* NCYC 366 that had been grown in defined medium and harvested in the mid-exponential phase of growth.

Values indicated are the means of four determinations.

Vertical bars indicate 95% confidence limits.

The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

Reagent	Percent PDS released after				
Trypsin	2h	4h	6h	8h	
	71.4	81.0	78.6	76.2	
β(1-3) glucanase	5 min	10 min	15 min	20 min	
	52.4	52.4	64.3	50.0	
KOH (6%, w/v)	0.25h	0.5h	1h	2h	3h
	71.4	83.3	85.7	76.2	71.4

Table 3. Release of PDS from PDS-saturated *Saccharomyces cerevisiae* NCYC 366 by treatment with trypsin, KOH (6%, w/v) or β(1-3) glucanase .

The organisms were grown in defined medium and harvested in the mid-exponential phase of growth.

COMPOSITION OF WALLS OF *SACCHAROMYCES CEREVISIAE* NCYC 366 BEFORE AND AFTER TREATMENT WITH DIFFERENT REAGENTS

Isolated walls of *Saccharomyces cerevisiae* NCYC 366 were analysed for glucan, mannan, protein and phosphorus before and after treatment with trypsin, β(1-3) glucanase, potassium hydroxide (6%, w/v) or hydrofluoric acid (58 - 62%, v/v), using the methods described for whole cells. These experiments were carried out to establish which cell-wall components were removed by the treatments, which were also used to treat whole cells in PDS-binding studies. The results are given in Tables 4 and 5. Glucan, mannan, protein and phosphorus accounted for about 84% of the dry weight of walls of *Saccharomyces cerevisiae* NCYC 366. The rest of the wall is most probably made up of lipid and N-acetylglucosamine.

Treatment of walls with trypsin removed most of the mannan, protein and phosphorus, but the glucan component remained unchanged. After treatment for 6 hours 95% of the mannan, 93% of the protein and 88% of the phosphorus had been removed (Table 4). Treatment of walls with β-glucanase removed mostly glucan with varying amounts of mannan, protein and phosphorus (Table 5). After treatment for 10 minutes 51% of the glucan, 5.7% of the mannan, 49% of the protein and 44% of the phosphorus were removed from the walls. Treatment with potassium hydroxide (6%, w/v) had a similar effect to trypsin treatment (Table 5). Most of the mannan, protein and phosphorus were removed with this treatment, although the glucan content was very little affected. After treatment for 30 minutes 95% of the mannan,

Component	Time of trypsin treatment (h)			
	0	2	4	6
Glucan (mg)	34.40 ± 4.1	33.8 ± 4.5	33.6 ± 4.2	33.7 ± 4.1
Mannan (mg)	41.60 ± 4.1	21.96 ± 4.5	9.0 ± 4.2	2.25 ± 4.1
Protein (mg)	7.0 ± 0.15	1.44 ± 0.17	0.9 ± 0.1	0.45 ± 0.12
Phosphorus (mg)	1.0 ± 0.05	0.43 ± 0.05	0.24 ± 0.07	0.12 ± 0.04
Weight of cell wall residue (mg)	100	72.0	60.2	48.0

Table 4 Composition of isolated walls of *Saccharomyces cerevisiae* NCYC 366 before and after treatment with trypsin for different periods of time. The values indicated are the means of four determinations ± 95% confidence limits. Organisms were grown in defined medium and harvested in the mid-exponential phase of growth. Confidence limits of glucan and mannan refer to the total polysaccharide content. Statistical tests for significance of difference were not carried out.

Component	Treatment			
	None	β(1-3)glucanase (10 min)	KOH (6%, w/v) (30 min)	HF (58-62%, v/v) 4h
Glucan (mg)	34.4 ± 4.1	16.8 ± 5.0	34.37 ± 4.0	33.0 ± 5.1
Mannan (mg)	41.6 ± 4.1	39.2 ± 5.0	2.25 ± 4.0	39.2 ± 5.1
Protein (mg)	7.0 ± 0.15	3.6 ± 0.15	1.65 ± 0.16	7.0 ± 0.26
Phosphorus (mg)	1.0 ± 0.05	0.56± 0.05	0.1 ± 0.06	0.1 ± 0.01
Weight of cell wall residue (mg)	100	70	50	91

Table 5 Composition of isolated walls of *Saccharomyces cerevisiae* NCYC 366 before and after treatment with β glucanase, KOH (6%, w/v) and hydrofluoric acid (58 - 62%, v/v). Values quoted are the means of four determinations ± 95% confidence limits. Organisms were grown in defined medium and harvested in the mid-exponential phase of growth. Confidence limits of glucan and mannan refer to the total polysaccharide content. Statistical tests for significance of difference were not carried out.

76% of the protein and 90% of the phosphorus were removed. Treatment of walls with hydrofluoric acid (58 - 62%, v/v) removed most of the phosphorus with little effect on the contents of other components (Table 5).

The mannan and phosphorus contents of walls after treatment with trypsin for different periods of time were related to the saturation concentration of PDS bound by whole cells of *Saccharomyces cerevisiae* NCYC 366 subjected to the same treatment (Figs. 20 and 21). The saturation concentration was found to be directly proportional to the mannan content and the phosphorus content of the wall.

SURFACE PROPERTIES OF *SACCHAROMYCES CEREVISIAE* NCYC 366 BEFORE AND AFTER TREATMENT WITH DIFFERENT REAGENTS

Properties of the cell surface of *Saccharomyces cerevisiae* NCYC 366 were investigated, before and after treatment with trypsin, β(1-3) glucanase, potassium hydroxide (6%, w/v) or hydrofluoric acid (58 - 62%, v/v), for any modification in the surface structure caused by the treatments.

Electrophoretic Mobility

The pH-mobility curves of yeast cells are described by the composition of the cell-wall components. Eddy & Rudin (1958a, b) postulated the presence of three types of ionizable groups responsible for the surface charge of yeasts. Of these, the phosphodiester groups of the phosphomannan have a constant effect on

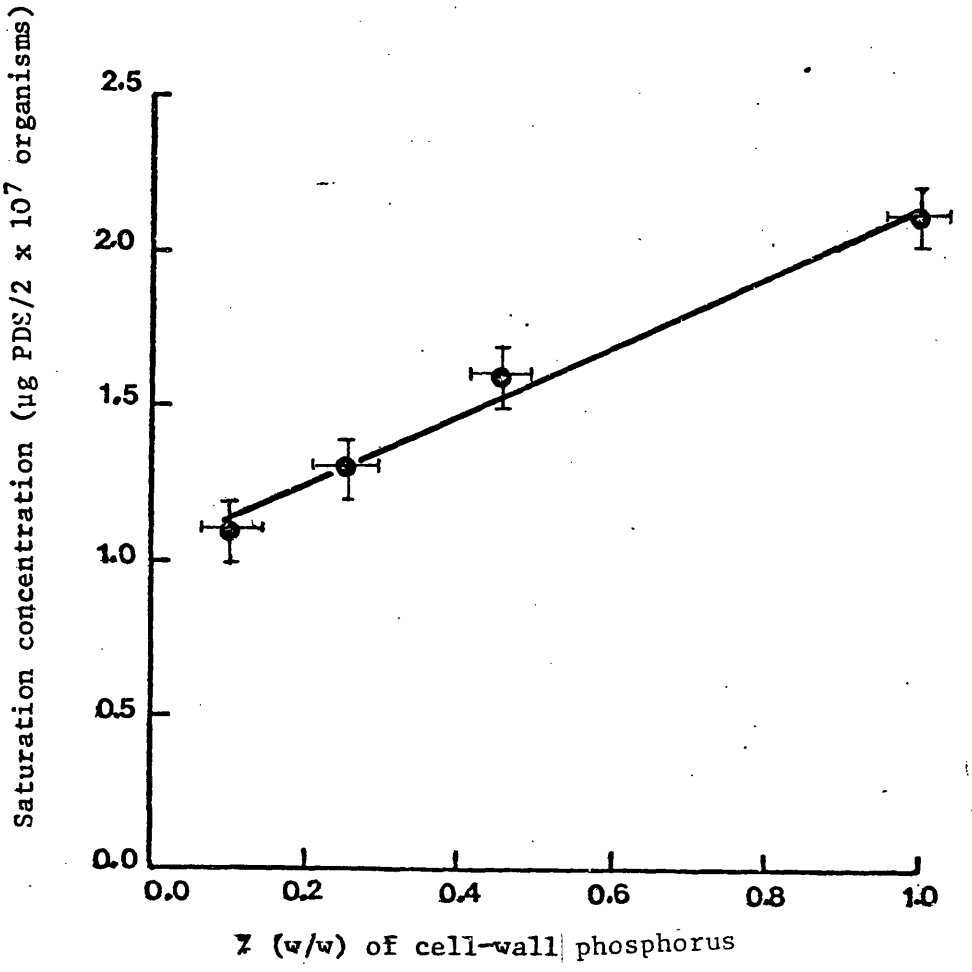


Figure 20 Relationship of cell-wall phosphorus content to the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366 grown in defined medium and harvested in the mid-exponential phase of growth. Phosphorus contents of the walls were changed by trypsin treatment.
Values indicated are the means of four determinations.
The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7
Correlation coefficient for this line was not calculated.

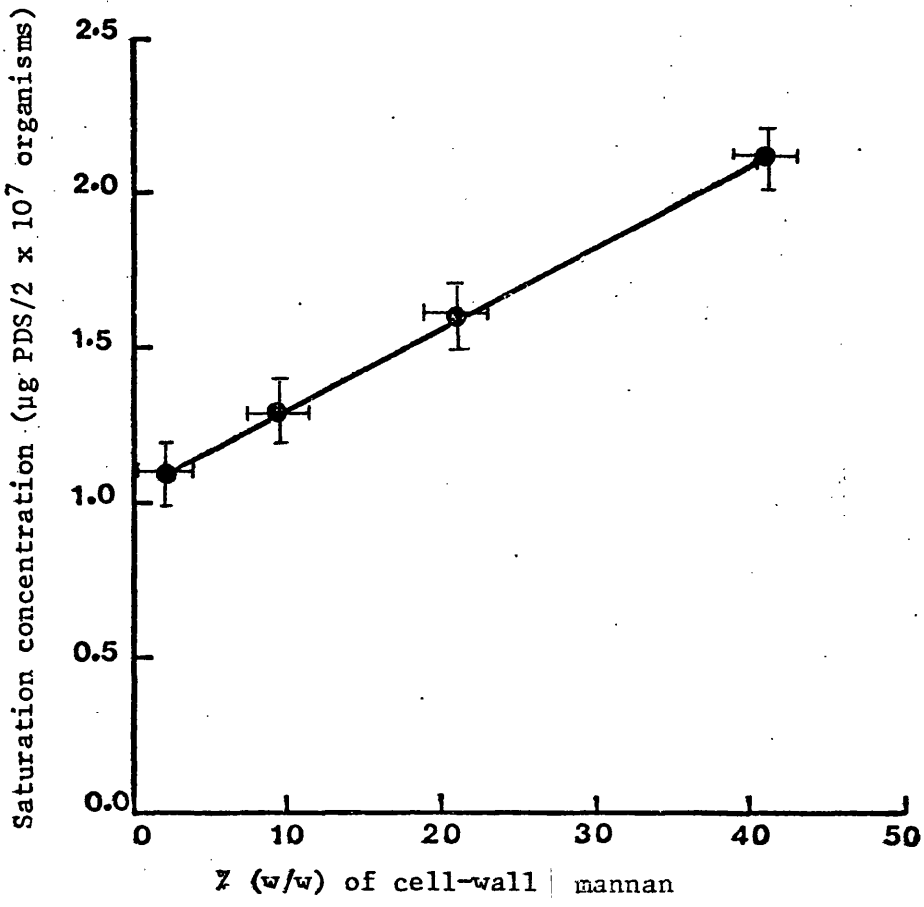


Figure 21 Relationship of cell-wall mannan content to the saturation concentration of PDS bound by *Saccharomyces* NCYC 366, that had been grown in defined medium and harvested in the mid-exponential phase of growth. Mannan contents of the walls were changed by trypsin treatment.

Values indicated are the means of four determinations.

The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

Correlation coefficient for this line was not calculated.

the negative mobility throughout the pH range 3 - 9, and a measure of the contribution to the overall mobility from these groups is given by the mobility value at pH 4.0 ($\mu_{4.0}$). The carboxyl groups of the acidic amino acids in the protein component are ionized only above pH 4.0, and a positive charge below pH 4.0 is due to protonated amino groups of the surface protein. Mobility due to surface protein is evident from a sharp change in value between pH 3.0 and pH 7.0 ($\mu_{7.0} - \mu_{3.0}$). Therefore, from the shape of the pH-mobility curves information regarding the composition of the wall surface can be obtained. Furthermore, by lowering the ionic strength of the buffer in which the mobility is measured, mobility due to ionizable groups in the deeper layers of the cell surface can be assessed. Fisher (1975) stated that the thickness of the surface layer on which the electrophoretic mobility depends approximate to 1.4 nm in buffers of ionic strength 0.05. By lowering the ionic strength of the buffer to 0.005 it is possible to make investigations of surface layers up to a depth of 4.2 nm.

Electrophoretic mobility of *Saccharomyces cerevisiae* NCYC 366 in the pH range 1.5 - 8.0 was studied in buffers of ionic strength 0.05 at 30°C, before and after treatment with trypsin, β-glucanase, potassium hydroxide (6%, w/v) or hydrofluoric acid (58 - 62., v/v; Fig. 22). The contributions from phosphate groups ($\mu_{4.0}$) and protein ($\mu_{7.0} - \mu_{3.0}$) are given in Table 6.

The pH mobility curve of untreated *Saccharomyces cerevisiae* NCYC 366 was typical of a surface with phsophate and protein, with the phosphate groups contributing a major portion towards the total

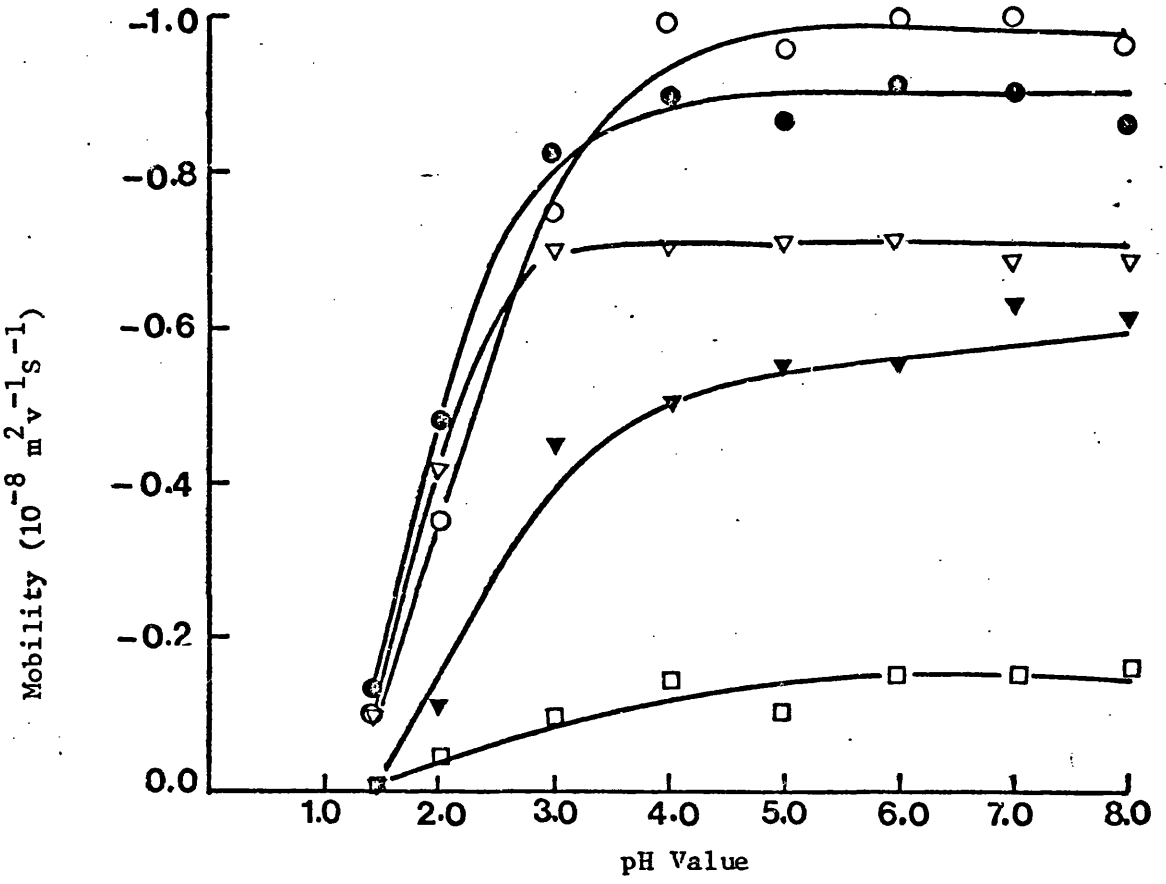


Figure 22 pH-Electrophoretic mobility curves of *Saccharomyces cerevisiae* NCYC 366 grown in defined medium and harvested in the mid-exponential phase of growth. ●—● untreated organisms ▽—▽ trypsin-treated (6 h) organisms, ▼—▼ KOH (6%, w/v)-treated (30 min) organisms, O—O β(1-3) glucanase-treated (10 min) organisms □—□ HF (58 - 62%, v/v)-treated (4h) organisms. Values indicated are the means of 20 observations. The standard error of the means was less than 2%. Measurements carried out in buffers of ionic strength 0.05.

Treatment	Mobility ($10^{-8} m^2 v^{-1} s^{-1}$) due to phosphate ($\mu_{4.0}$)	protein ($\mu_{7.0} - \mu_{3.0}$)
None	0.89	0.095
Trypsin (6 h)	0.70	0.010
β(1-3) glucanse (10 min)	0.94	0.225
KOH (6%, w/v ; 30 min)	0.50	0.17
HF (58 - 62%, v/v; 4 h)	0.13	0.06

Table 6. Electrophoretic mobility due to phsophate and protein of *Saccharomyces cerevisiae* NCYC 366 treated with different reagents. These values were calculated according to Eddy & Rudin (1958b). Organisms were grown in defined medium and harvested in the mid-exponential phase of growth.

mobility. Trypsin-treated cells showed a pH-mob ility pattern typical of a surface with a large amount of phosphate groups with only a small contribution from protein. However the mobility at pH 4.0, due to phosphate groups, showed a drop of about 20% as compared with untreated cells. The curve obtained for β-glucanase-treated cells revealed an increased exposure of charged groups due to the treatment. Both contributions due to phosphate and protein were higher than with untreated cells. A drop in the contribution from phosphate to the mobility greater than with trypsin-treated cells was observed with cells treated with potassium hydroxide (6%, w/v). However a higher contribution from protein was indicated due to the rapid increase in mobility from pH 3.0 - 7.0. Finally the hydrofluoric acid-treated cells were found to have a very low overall mobility.

Binding of Fluorescent Antibody

Binding of fluorescent antibody by micro-organisms depend on the presence of the appropriate antigenic groups on the cell surface. The principal immunogenic groups of *Saccharomyces cerevisiae* are associated with the mannan component of the wall. Therefore antibody raised against whole cells of *Saccharomyces cerevisiae* could be regarded as a reagent specific for the mannan of the wall. The intensity of fluorescent antibody binding will provide an estimate of the amount of surface mannan. However, modifications to the mannan structure such as by removal of immunogenic groups, could interfere with the antibody binding.

Antibody against whole cells of *Saccharomyces cerevisiae* NCYC 366 were raised and the binding of fluorescent antibody by cells before and after treatment with trypsin, β-glucanase, potassium hydroxide (6%, w/v) or hydrofluoric acid (58 - 62%, v/v) was studied. The intensities of antibody binding are given in Table 7. These intensities were estimated visually by microscopic examination using ultraviolet radiation.

Binding of fluorescent antibody by trypsin-treated organisms was low compared with untreated organisms, giving only faint fluorescence. However β-glucanase-treated organisms bound fluorescent antibody avidly showing very little difference in the intensity of fluorescence compared with untreated organisms. Binding of fluorescent antibody by cells treated with potassium hydroxide (6%, w/v) was comparable with that of cells treated with trypsin, whereas hydrofluoric acid-treated organisms showed very faint fluorescence indicating very little binding .

Photographic records of some of the preparations are shown in Plates 1 and 2. Other preparations could not be photographed due to the very weak fluorescence, which faded rapidly under ultraviolet radiation.

Binding of Fluorescent Concanavalin A

Concanavalin A, a phytohaemagglutinin isolated from jack bean *(Canavalia ensiformis)*, combines specifically with a variety of polysaccharides which have branched structures and α-D-glucopyranosyl, α-D-mannopyranosyl, β-D-fructofuranosyl or α-D-arabinofuranosyl

Treatment				
None	trypsin (6 h)	β-glucanase (10 min)	KOH (6%, w/v; 30 min)	HF (58-62%,v/v; (4h)
+++++	++	++++	++	+

Table 7. Binding of fluorescent antibody by treated and untreated *Saccharomyces cerevisiae* NCYC 366. Organisms were grown in defined medium and harvested in the mid-exponential phase of growth.

Key: +++++ very bright fluorescence, ++++ bright fluorescence, ++ faint fluorescence and + very faint fluorescence.

Plate 1 Fluorescent antibody stained preparation of *Saccharomyces cerevisiae* NCYC 366 (Magnification - x 2000)

Plate 2 Fluorescent antibody stained preparation of *Saccharomyces cerevisiae* NCYC 366 that had been pre-treated with β(1-3) glucanase for 10 min. (Magnification - x 2000)

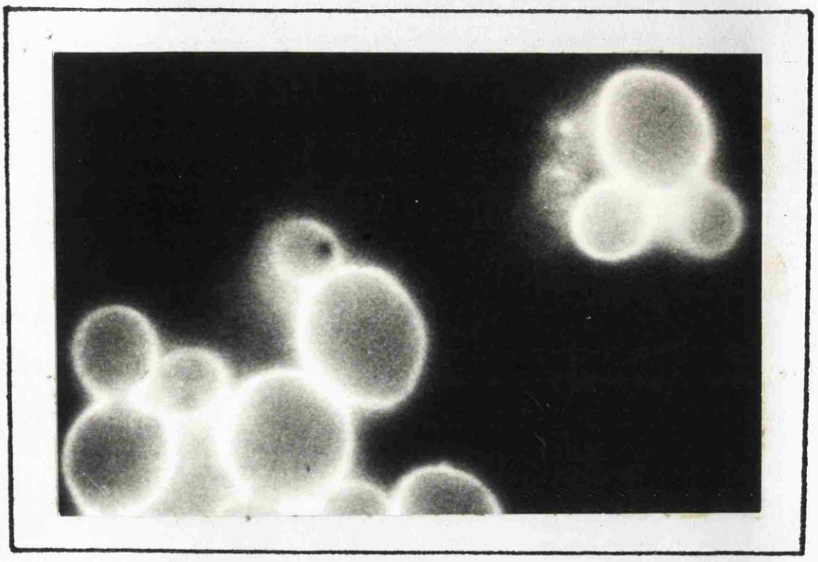


Plate 1

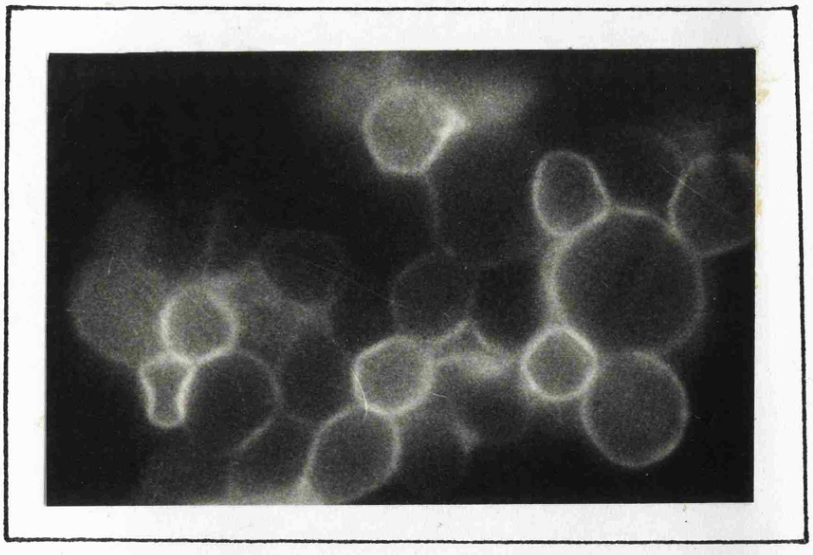


Plate 2

residues occupying non-reducing terminal positions. Of the polysaccharides which react with concanavalin A α-linked mannose-containing homopolymers such as those in the walls of *Saccharomyces* species, seem to be the most tenaciously bound. Since concanavalin A does not react with yeast wall glucan and would not be expected to react with chitin, a β-linked polymer, fluorescently labelled concanavalin A should be a specific reagent for α-mannan present in the walls of *Saccharomyces cerevisiae* (Tkacz *et al.* 1971).

Binding of fluorescent concanavalin A by *Saccharomyces cerevisiae* NCYC 366 before and after treatment with trypsin, β(1-3) glucanase, potassium hydroxide (6%, w/v) and hydrofluoric acid (58 - 62%, v/v) was studied. The intensities of binding was estimated visually by microscopic examination using ultraviolet radiation (Table 8).

Photographic records of the preparations are shown in Plates 3-7. Binding of fluorescent concanavalin A by organisms treated with trypsin or KOH (6%, w/v) was low compared with untreated organisms as estimated by intensity of fluorescence. Treatment of organisms with β-glucanase caused only a small change as far as binding of fluorescent concanavalin A was concerned. Organisms treated with hydrofluoric acid bound fluorescent concanavalin A more or less tc the same extent as untreated organisms.

Treatment				
None	trypsin (6h)	β-glucanase (10 min)	KOH (6%,w/v; 30 min)	HF(58-62%, v/v; 4h)
+++++	++	++++	++	+++++

Table 8 Binding of fluorescent concanavalin A by treated and untreated *Saccharomyces cerevisiae* NCYC 366

Organisms were grown in defined medium and harvested in the mid-exponential phase of growth.

Key: +++++ very bright fluorescence, ++++ bright fluorescence ++faint fluorescence.

Plate 3 Fluorescent concanavalin A stained preparation of *Saccharomyces cerevisiae* NCYC 366
(Magnification - x 2000)

Plate 4 Fluorescent concanavalin A stained preparation of *Saccharomyces cerevisiae* NCYC 366 that had been pre-treated with trypsin (6 h).
(Magnification - x 2000)

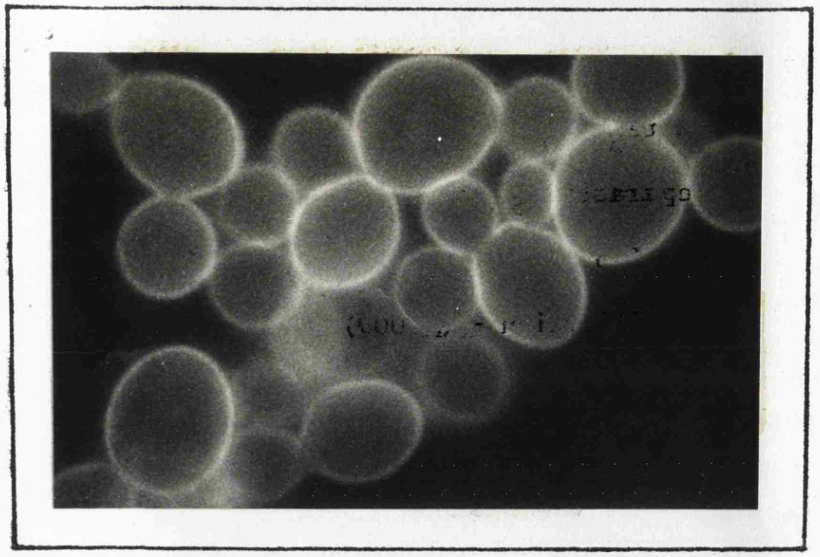


Plate 3

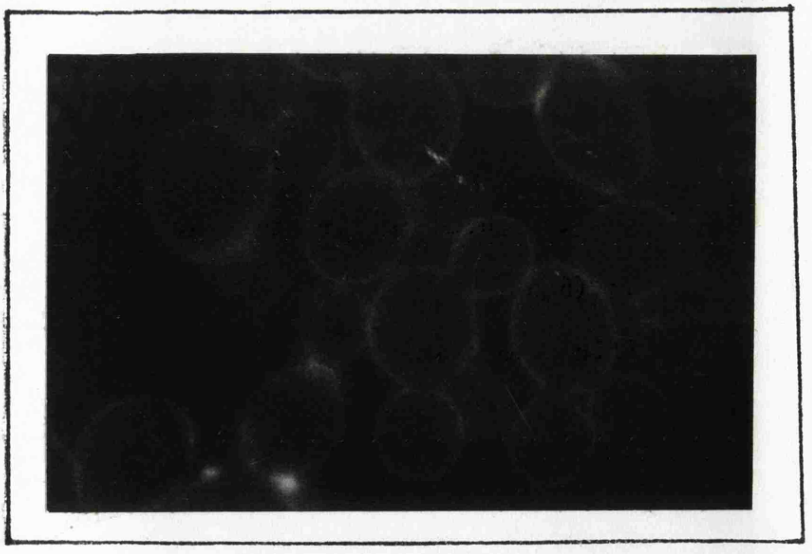


Plate 4

Plate 5 Fluorescent concanavalin A stained preparation of *Saccharomyces cerevisiae* NCYC 366 that had been pre-treated with β(1-3) glucanase (10 min)
(Magnification x 2000)

Plate 6 Fluorescent concanavalin A stained preparation of *Saccharomyces cerevisiae* NCYC 366 that had been pre-treated with KOH (6%, w/v; 30 min)
(Magnification x 2000)

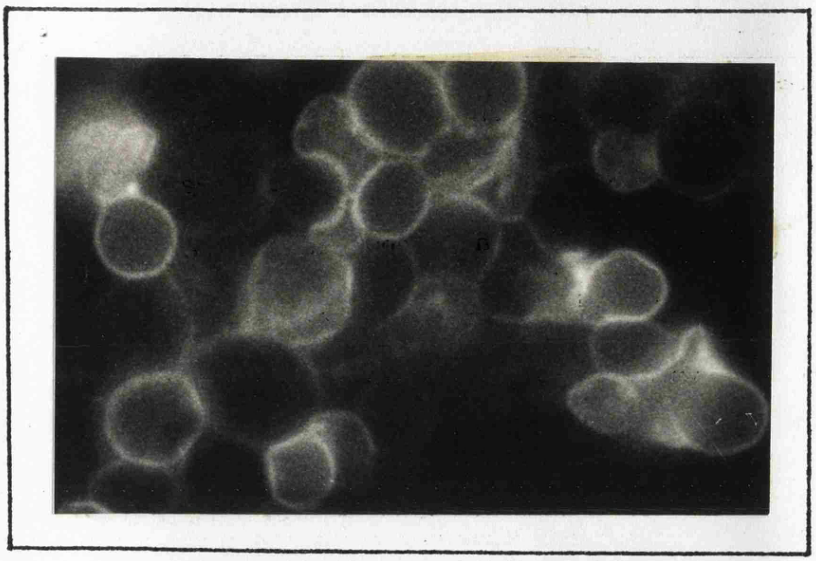


Plate 5

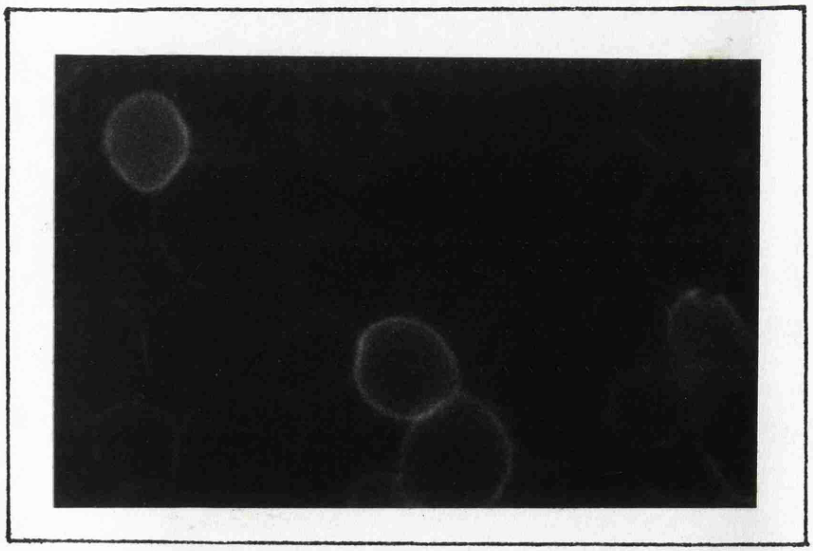


Plate 6

Plate 7 Fluorescent concanavalin A stained preparation of *Saccharomyces cerevisiae* NCYC 366 that had been pre-treated with hydrofluoric acid (58 - 62%, v/v; 4h) (Magnification - x 2000)

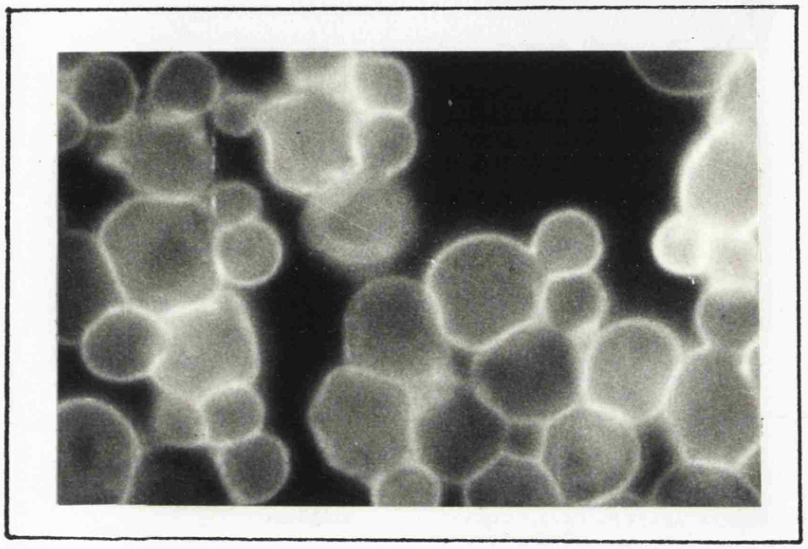


Plate 7

BINDING OF PDS BY *SACCHAROMYCES CEREVISIAE* NCYC 366 TREATED WITH CONCANAVALIN A

Concanavalin A is a reagent which reacts specifically with the mannan component of the yeast cell wall.

Studies were made on the binding of PDS by *Saccharomyces cerevisiae* NCYC 366 that had been treated with concanavalin A to investigate the effect of this treatment on PDS binding. Washed *Saccharomyces cerevisiae* NCYC 366 was suspended in 1M – NaCl containing 200 µg concanavalin A per ml at a concentration of 10 mg dry weight equivalent per ml. The suspension was incubated at 30°C for one hour on an orbital shaker (300 rev per min). The cells were separated by centrifuging and were washed once in distilled water. Binding of PDS was studied as described in the Methods section using Antifoam M-10 in 0.1M-KH_2PO_4 buffer (pH 4.5). Concanavalin A-treated cells were found to bind about 50% less PDS than the control. The rate of binding also was much slower (Figure 23).

BINDING OF PDS BY *SACCHAROMYCES CEREVISIAE* NCYC 366 CROWN IN MYGP MEDIUM

Since all experiments so far described on PDS binding by *Saccharomyces cerevisiae* NCYC 366 were carried out on organisms grown in defined medium, the effect of growth in nutritionally rich medium was studied by using organisms grown in MYGP medium (Wickerham, 1951). Organisms were harvested in the mid-exponential

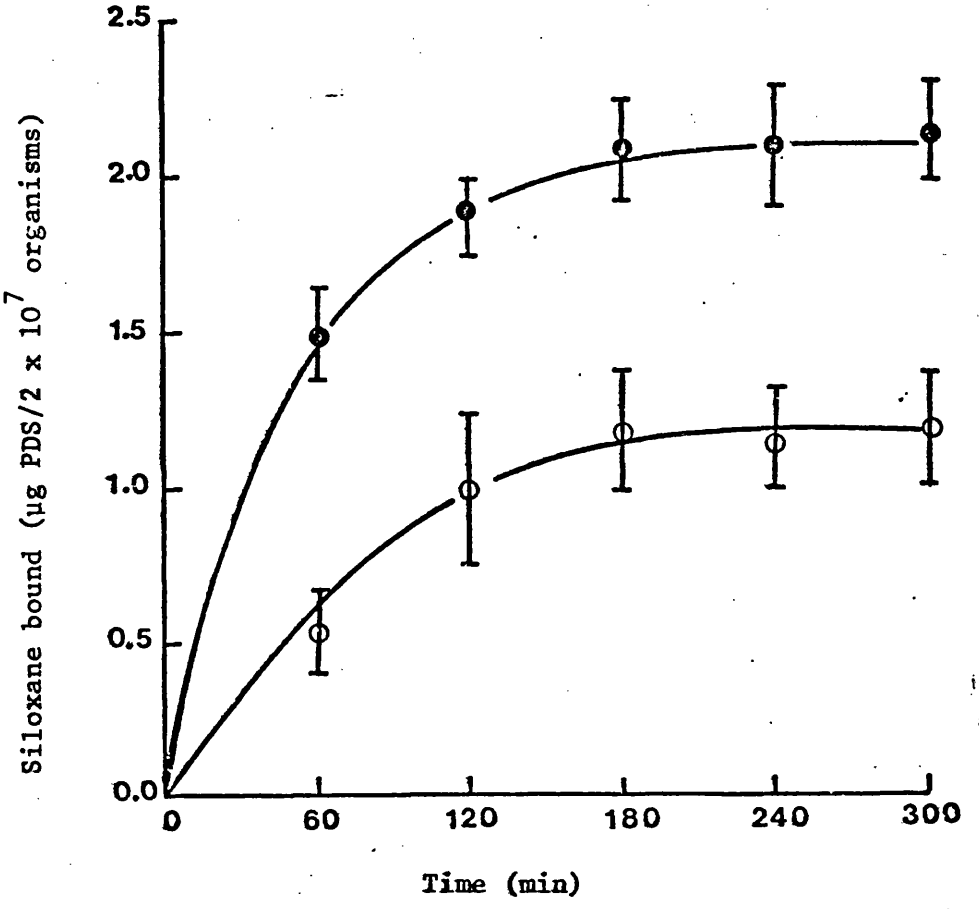


Figure 23 Time-course of PDS binding by *Saccharomyces cerevisiae* NCYC366 after treatment with concanavalin A. ●—● indicates untreated organisms, O—O concanavalin A-treated organisms, The organisms were grown in defined medium and harvested in the mid-exponential phase of growth. Values indicated are the means of four determinations. Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated *Saccharomyces cerevisiae* NCYC 366 is equal to 2×10^7.

phase of growth and washed in 0.1 M KH_2PO_4 buffer (pH 4.5) before studying binding of PDS. Binding of PDS was studied using Antifoam M-10 as described in the Methods section. Organisms grown in MYGP medium bound about 0.4 µg PDS per 2 x 10^7 organisms more than organisms grown in defined medium (Fig. 24).

Walls of organisms grown in MYGP medium contained higher contents of mannan, glucan and phosphorus (Table 9).

EFFECT OF THE SURFACE CHARGE OF *SACCHAROMYCES CEREVISIAE* NCYC 366 ON PDS BINDING

The pH-electrophoretic mobility curve of *Saccharomyces cerevisiae* NCYC 366 (Fig. 22) shows that the surface charge carried by the cells is more or less constant above pH 4.0 but diminishes rapidly below this value. The effect of the charge on PDS binding by these was investigated by studying binding at pH values lower than 4.0. The procedure used was the same as that described in the Methods section but Sørenson's glycine I (Sørenson, 1909, 1912) buffer solutions of pH 3.5, 2.5 and 1.5, containing Antifoam M-10 were used instead of 0.1M - KH_2PO_4.

The results (Table 10) show that the surface charge has no obvious effect on the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC366.

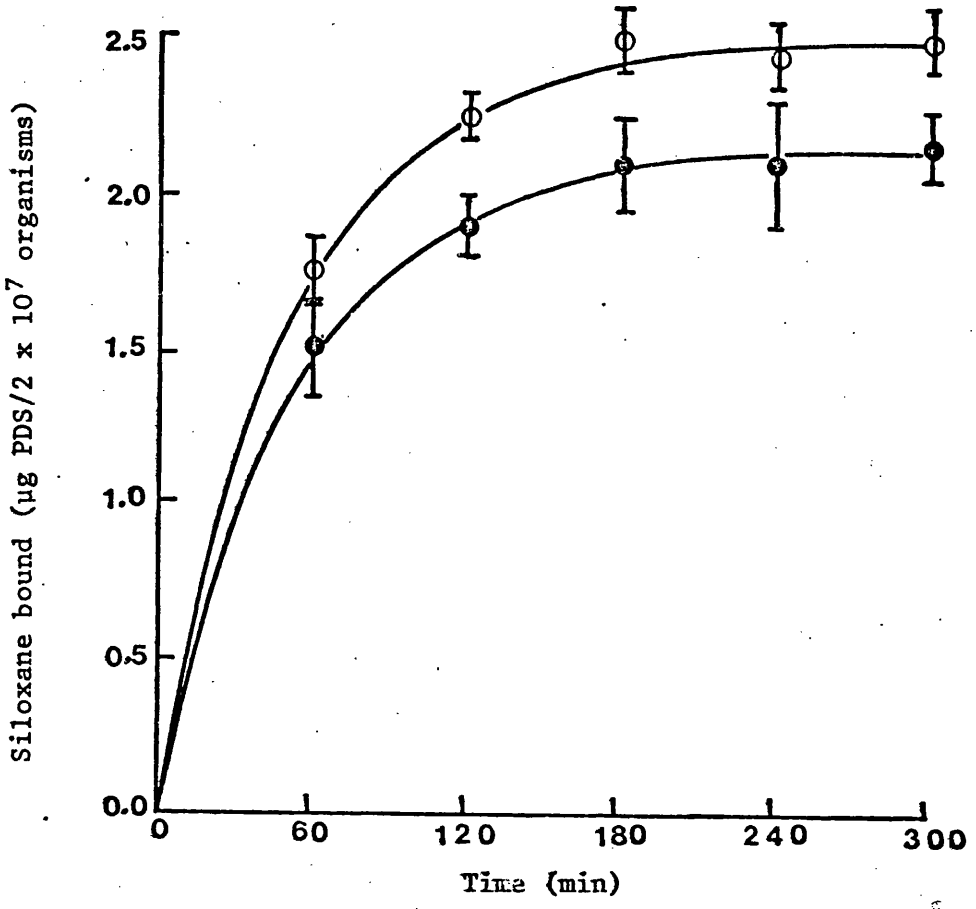


Figure 24. Binding of PDS by *Saccharomyces cerevisiae* NCYC 366 grown either in defined medium (●—●) or in MYGP medium (O—O) (Wickerham, 1951). All organisms are harvested in the mid-exponential phase of growth. Values indicated are the means of four determinations. Vertical bars indicate 95% confidence limits. The number of organisms in one mg dry weight equivalent of untreated cells of *Saccharomyces cerevisiae* NCYC 366 is equal to 2 x 10^7.

Component	Defined medium	MYGP medium
Glucan %	34.4 ± 4.1	36.04 ± 5.2
Mannan %	41.6 ± 4.1	43.96 ± 5.2
Protein %	7.0 ± 0.15	7.1 ± 0.26
Phosphorus %	1.0 ± 0.05	1.2 ± 0.05

Table 9. Composition of walls of *Saccharomyces cerevisiae* that had been grown in defined medium or MYGP medium (Wickerham, 1951) and harvested in the mid-exponential phase of growth. Confidence limits of glucan and mannan refer to the total polysaccharide content. Statistical tests for significance of difference were not carried out.

pH value	Mobility value ($10^{-8} m^2 V^{-1} S^{-1}$)	Saturation Concentration (μg PDS/2 x 10^7 organisms)
1.5	-0.13	2.00 ± 0.09
2.5	-0.50	1.95 ± 0.11
3.5	-0.86	2.00 ± 0.08
4.5	-0.90	2.10 ± 0.10

Table 10 Effect of pH value of the suspending medium on the surface charge and the saturation concentration of PDS bound by *Saccharomyces cerevisiae* NCYC 366 that had been grown in defined medium and harvested in the mid-exponential phase of growth.

EFFECT OF PDS BINDING ON SOME SURFACE PROPERTIES OF *SACCHAROMYCES CEREVISIAE* NCYC 366

The effect of PDS-binding on the electrophoretic mobility, binding of fluorescent antibody, binding of fluorescent concanavalin A and invertase activity of *Saccharomyces cerevisiae* NCYC 366 was studied. Organisms were saturated with PDS by suspending them at 2.0 mg dry weight equivalent per ml in 0.1M - KH_2PO_4 buffer (pH 4.5) containing Antifoam M-10 emulsion (200 µg per ml) and incubating on an orbital shaker (300 rev per min) at 30°C for 4 hours.

Electrophoretic Mobility

Organisms saturated with PDS showed a decreased mobility between pH 4.0 and 8.0 as compared with untreated organisms (Fig. 25). Mobility due to phosphate groups ($\mu_{4.0}$) of organisms saturated with PDS was lower by about 11% compared with untreated organisms. The calculated mobility due to protein ($\mu_{7.0} - \mu_{3.0}$) of organisms saturated with PDS was negative (Table 11).

Binding of Fluorescent Antibody and Concanavalin A

Binding of both antibody raised against whole cells and of concanavalin A by *Saccharomyces cerevisiae* is with the mannan component of the cell wall . To investigate any masking effect on the mannan caused by saturation of organisms with PDS, binding of fluorescent antibody and fluorescent concanavalin A by

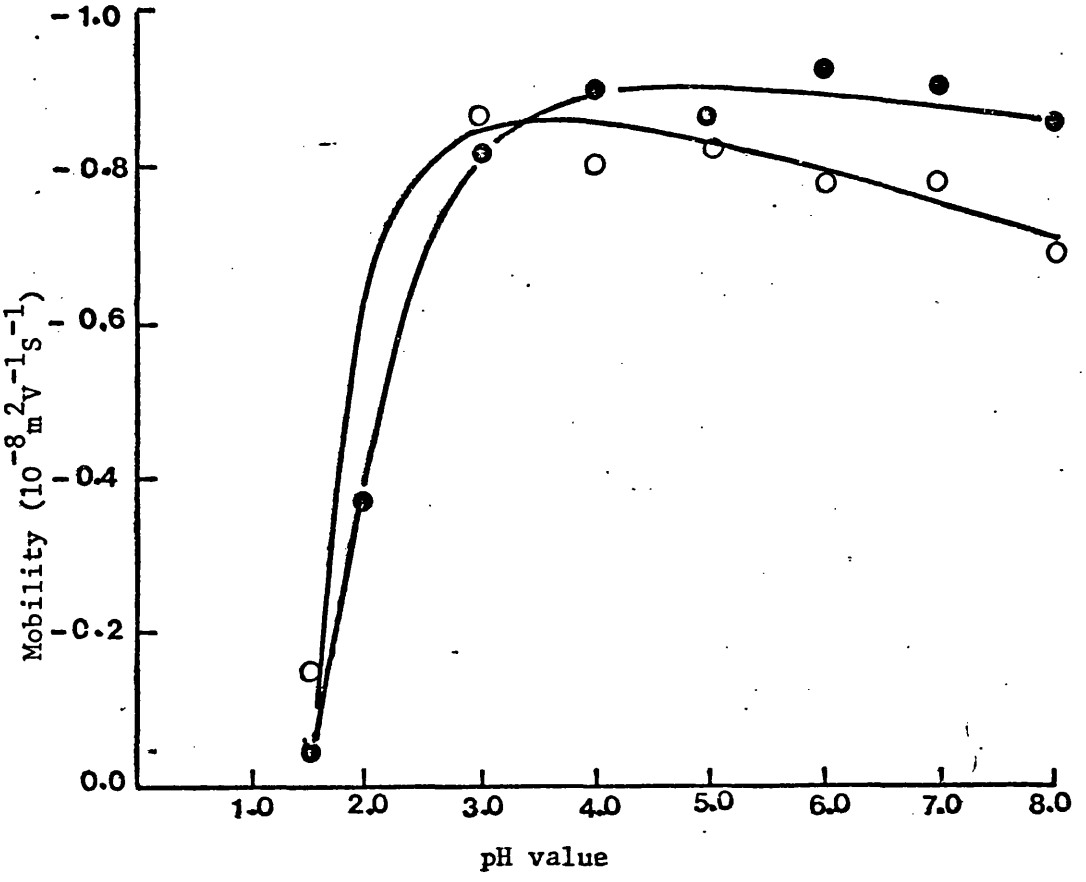


Figure 25 pH-Electrophoretic mobility curves of *Saccharomyces cerevisiae* NCYC 366, that had been grown in defined medium and harvested in the mid-exponential phase of growth. ●—● indicates untreated organisms, O—O organisms treated with PDS. Values indicated are the means of 20 observations. Standard error of the mean was less than 2%.

Organisms	Mobility ($10^{-8} m^2 V^{-1} S^{-1}$) due to	
	Phosphate ($\mu_{4.0}$)	Protein ($\mu_{7.0} - \mu_{3.0}$)
Untreated	0.9	0.09
PDS-saturated	0.8	-0.08

Table 11 Mobilities due to phosphate ($\mu_{4.0}$) and protein ($\mu_{7.0} - \mu_{3.0}$) of *Saccharomyces cerevisiae* NCYC 366, before and after saturation with PDS. Organisms were grown in defined medium and harvested in the mid-exponential phase of growth.

Saccharomyces cerevisiae NCYC 366 was studied before and after saturation with PDS. The intensities of binding were estimated visually under the microscope using ultraviolet radiation (Table 12). A small but consistent drop in the intensity of binding both fluorescent antibody and fluorescent concanavalin A was observed with organisms saturated with PDS as compared with untreated organisms. Photographic records of fluorescent antibody and fluorescent concanavalin A stained preparations of PDS-saturated *Saccharomyces cerevisiae* NCYC 366 are shown in plates 8 and 9.

Invertase Activity

The invertase activity of *Saccharomyces cerevisiae* is associated with a glycoprotein in the cell wall which contains about 50% of mannan. The effect of PDS binding on the release of this enzyme by *Saccharomyces cerevisiae* NCYC 366 was studied. The invertase activity was estimated by a method based on that described by Sutton & Lampen (1962). The results showed that saturation of the organisms with PDS has no effect on the invertase activity (Table 13).

Binding reagent	Organisms untreated	PDS-saturated
Fluorescent Antibody	+++++	+++
Fluorescent Concanavalin A	+++++	+++

Table 12 Binding of fluorescent antibody and fluorescent concanavalin A by *Saccharomyces cerevisiae* NCYC 366 before and after saturation with PDS. Organisms were grown in defined medium and harvested in the mid-exponential phase of growth.

Key: +++++ Very bright fluorescence;

+++ Moderate fluorescence.

Plate 8 Fluorescent antibody stained preparation of *Saccharomyces cerevisiae* NCYC 366 that had been pre-treated with 0.1M KH_2PO_4 buffer (pH 4.5) containing Antifoam M-10 (200 µg per ml) for 4h. (Magnification - x 2000)

Plate 9 Fluorescent concanavalin A stained preparation of *Saccharomyces cerevisiae* NCYC 366 that had been pre-treated with 0.1 M KH_2PO_4 buffer (pH 4.5) containing Antifoam M-10 (200 µg per ml) for 4 h. (Magnification - x2000

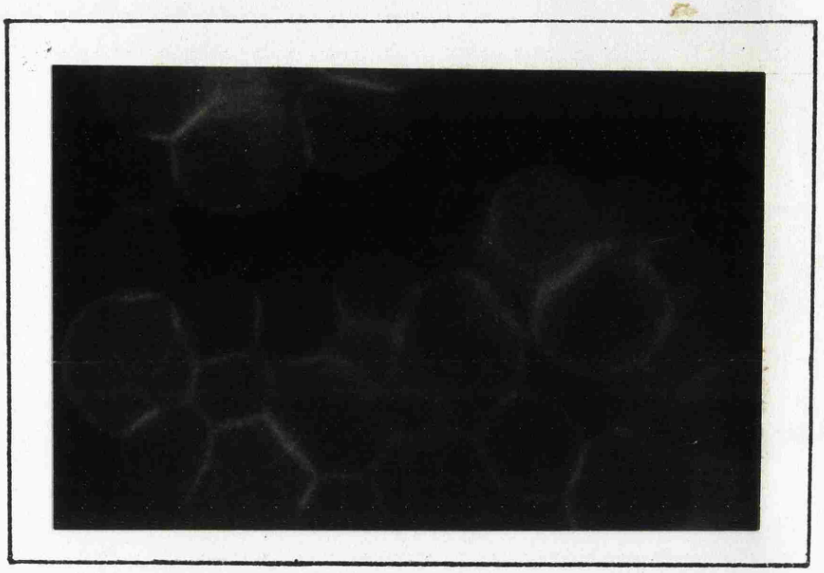


Plate 8

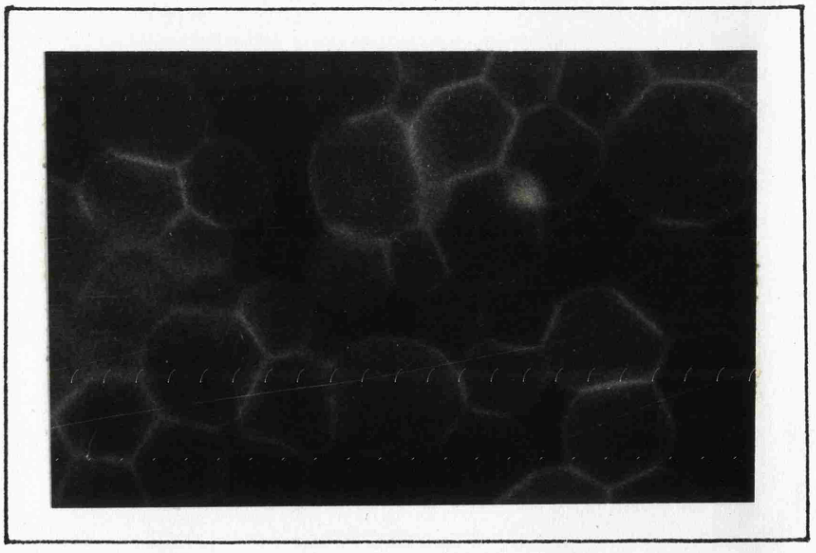


Plate 9

	Organisms	
	Untreated	PDS-saturated
Invertase activity (μM sucrose consumed per mg dry weight equivalent per min)	0.35 ± 0.03	0.34 ± 0.04

Table 13. Effect of PDS saturation on the invertase activity of *Saccharomyces cerevisiae* NCYC 366. Values indicated are the means of four determinations ± 95% confidence limits. Organisms were grown in defined medium and harvested in the mid-exponential phase of growth.

PART II

ROLE OF PHOSPHODIESTER-LINKED MANNAN IN FLOCCULATION OF *SACCHAROMYCES CEREVISIAE*

EFFECT OF HYDROFLUORIC ACID TREATMENT ON THE CELL-WALL COMPOSITION OF *SACCHAROMYCES CEREVISIAE*

Treatment of phosphomannan, extracted from cell-walls of *Saccharomyces cerevisiae* with hydrofluoric acid (40 %, v/v) at room temperature has been reported to remove 70% of the phosphorus without any detectable decrease in the molecular weight (Cawley *et al.*, 1972). Hydrofluoric acid (60%, v/v) has also been used to cleave phosphodiester groups in teichoic acid (Burger & Glaser, 1964; Archibald *et al.*, 1968) and phospholipids (Shaw & Stead, 1974) extracted from bacteria. Because concentrated hydrofluoric acid can remove phosphate groups, in the diesterified state, from compounds including polysaccharides,without any significant hydrolysis of glycosidic bonds, it was used to treat cells and walls of *Saccharomyces cerevisiae* in order to remove phosphate groups from the wall phosphomannan.

Isolated walls of *Saccharomyces cerevisiae* NCYC 366, NCYC 1004, NCYC 1005 and NCYC 1063 were analysed for glucan, mannan, protein and phosphorus before and after treatment with hydrofluoric acid (58 - 62%, v/v). Walls from each of the strains of *Saccharomyces cerevisiae* used in this study differed slightly in their contents of glucan, mannan, protein and phosphorus (Table 14). These components accounted for 84 - 91% of the dry weight of the walls, the remainder of the wall presumably being accounted for by lipid and small amounts of glucosamine neither of which was determined in this study. Treating walls with hydrofluoric acid led to the loss of 7 - 10% of the dry weight. About half of this loss can

Component	NCYC 366		NCYC 1004		NCYC 1005		NCYC 1063	
	untreated	HF-treated	untreated	HF-treated	untreated	HF-treated	untreated	HE-treated
Glucan (mg)	35.04±4.4	33.95±5.1	36.45±4.6	35.94±3.9	40.80±4.0	38.80±4.6	46.90±4.5	44.70±4.2
Mannan (mg)	44.96±4.4	42.01±5.1	44.55±4.6	41.06±3.9	44.20±4.0	41.20±4.6	31.10±4.5	28.30±4.2
Protein (mg)	7.00±0.31	6.91±0.28	6.30±0.31	6.00±0.28	7.56±0.17	7.50±0.20	12.10±0.35	10.0 ±0.31
Phosphorus (mg)	1.25±0.01	0.15±0.015	0.40±0.017	0.08±0.01	0.50±0.06	0.08±0.005	0.70±0.01	0.10±0.011
Weight of sample	100.0	91.0	100.0	91.0	100.0	93.0	100.0	90.0

Table 14. Effect of hydrofluoric acid (58 - 62%, v/v) treatment on composition of isolated walls of strains of *Saccharomyces cerevisiae*. Values quoted are the means of four determinations ±95% confidence limits. Organisms were grown in MYGP medium and harvested in the stationary phase of growth. Confidence limits of glucan and mannan refer to the total polysaccharide content. Statistical tests for significance of difference were not carried out.

be accounted for by the disappearance of a large proportion of the phosphorus in the wall, and by the loss of some mannan and a little glucan. Walls of strain NCYC 1063 also lost some protein following treatment with hydrofluoric acid. The decrease in phosphorus (about 80 - 90%) and mannan (about 6 - 9%) contents indicate that treatment with hydrofluoric acid leads to the removal of most of the phosphodiester linkages and release presumably mannose oligosaccharides which lie terminal to the phosphodiester linkages.

EFFECT OF HYDROFLUORIC ACID TREATMENT ON THE SEDIMENTATION RATES OF *SACCHAROMYCES CEREVISIAE*

Since treatment of walls of *Saccharomyces cerevisiae* with hydrofluoric acid removed 80 - 90% of the wall phosphorus without causing extensive losses of other components, this treatment was used on whole cells of *Saccharomyces cerevisiae* in order to study the effect of removal of phosphorus from wall phosphomannan on flocculation. Freeze-dried whole cells of *Saccharomyces cerevisiae* were treated with hydrofluoric acid (58 - 62%, v/v) and the rate of flocculation as expressed by their sedimentation rates was studied before and after the treatment. In a separate set of experiments it was established that the sedimentation rates of freeze-dried organisms were comparable to those of freshly harvested organisms. Sedimentation rates were measured at pH 4.5 either in 0.05M sodium acetate buffer supplemented with $CaCl_2$ (0.1%, w/v) or in deionized water, as flocculence is best expressed in the range pH 4.5 - 5.5 (Mill, 1964b). Sedimentation rates of untreated and

hydrofluoric acid-treated cells of *Saccharomyces cerevisiae* NCYC 366, NCYC 1004, NCYC 1005 and NCYC 1063 are given in Table 15.

Of the four strains studied, untreated cells of NCYC 366 and NCYC 1004 had very low sedimentation rates when tested in $CaCl_2$ supplemented sodium acetate buffer (pH 4.5), characteristic of non-flocculent yeast, whereas NCYC 1005 and NCYC 1063 showed much higher sedimentation rates characteristic of flocculent yeasts. Treatment with hydrofluoric acid caused the sedimentation rates of all four strains to increase, the increase being greater with the two non-flocculent strains (NCYC 366 and NCYC 1004). Sedimentation rates of untreated cells of the two flocculent strains (NCYC 1005 and NCYC 1063) declined to very low values characteristic of non-flocculent strains when the cells were washed and sedimentation rates were tested in deionized water. Although the sedimentation rates of hydrofluoric acid-treated cells of all four strains also declined to low values with the same treatment, they needed to be washed with 10 mM EDTA for their sedimentation rates to decline to values characteristic of non-flcoculent strains. Organisms deflocculated by washing in deionized water or 10 mM EDTA regained flocculence following the addition of $CaCl_2$ (0.1%, w/v) into the suspending medium.

There was a possibility that at least some of the effects observed following treatment of yeasts with hydrofluoric acid were caused by retention of F^- ions in the envelope layers of the organisms. Assays for fluoride were impracticable mainly because of the presence of appreciable amounts of phosphate in yeast walls.

Secondary treatment	Flocculation medium	Sedimentation Rate (µg dry weight equivalent per ml per min). NCYC 366		NCYC 1004		NCYC 1005		NCYC 1063	
		Untreated	HF-treated	Untreated	HF-treated	Untreated	HF treated	Untreated	HF-treated
None	Sodium acetate (0.05M) buffer (pH 4.5) containing $CaCl_2$ (0.1%, w/v)	165±12	1846±26	120±7	1666±11	1764±11	3000±21	1200±13	2181±12
Washed in deionized water	Deionized water	-	600±22	-	400±8	100±6	420±11	100±5	360±7
Washed in 10 mM EDTA	Deionized water	-	150±6	-	128±9	-	90±7	-	120±6

Table 15. Sedimentation rates of *Saccharomyces cerevisiae*. The values quoted are the means of four determinations ± 95% confidence limits. Organisms were grown in MYGP medium (Wickerham, 1951) and harvested in the stationary phase of growth. Treatment with hydrofluoric acid was carried out as described in Methods Section.

Untreated organisms were therefore suspended in a solution of potassium fluoride (11%, w/v), which had the same F^- ion concentration as in the hydrofluoric acid treatment, for 5 h and the sedimentation rates of washed organisms were measured in $CaCl_2$-supplemented sodium acetate buffer (0.05M, pH 4.5). This treatment had no measurable effect on the sedimentation rates of any of the strains except NCYC 1005, where the rate declined to about half of that of organisms that had not been treated (Table 16).

Analyses of hydrofluoric acid-treated walls show the loss of components other than those analysed. In order to assess the effect of extracting lipids, freeze-dried cells were extracted by the method of Letters (1968) modified by Hunter & Rose (1972). Treated cells were washed and freeze-dried, and the sedimentation rates of these cells measured in 0.05M sodium acetate buffer (pH 4.5) containing $CaCl_2$ (0.1%, w/v). Cells of non-flocculent strains showed more or less the same sedimentation rates as the corresponding untreated cells whereas flocculent strains showed much lower sedimentation rates following lipid extraction (Table 16).

Treatment of yeast with 1,2 epoxypropane (5%, v/v) in 0.05M sodium acetate buffer (pH 4.5), which is an esterifying agent, has been claimed to esterify carboxyl groups in the cell wall (Mill, 1964b) and cause a drop of 20% in the calcium-binding capacity of yeast cell walls (Lyons & Hough, 1970a, b). It was also claimed by Mill (1964b) that this treatment caused complete deflocculation of potentially flocculent yeast. However in the present study, although a considerable decrease in sedimentation

Secondary Treatment	Flocculation medium	Sedimentation Rate (µg dry weight equivalent per ml per min)							
		NCYC 366		NCYC 1004		NCYC 1005		NCYC 1063	
		Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated
None	Sodium acetate 0.05M buffer (pH 4.5) containing $CaCl_2$ (0.1%, w/v)	165±12	1846±26	120±7	1666±11	1764±11	3000±21	1200±13	2181±12
Treated with KF (11%,w/v) for 5 h.	----do-----	151±7	-	124±5	-	925±23	-	1065±25	-
Lipid extracted	----do-----	148±9	-	132±8	-	540±17	-	200±11	-
Treated with 1,2-epoxypropane (5%,v/v)	do	-	1153±38	-	705±7	880±11	705±7	675±7	461±6

Table 16. Effect of different treatments with or without treatment with hydrofluoric acid, on the sedimentation rates of strains of *Saccharomyces cerevisiae*. Values quoted are the means of four determinations ± 95% confidence limits. Organisms were grown in MYGP medium (Wickerham, 1951) and harvested in the stationary phase of growth Treatment with hydrofluoric acid was carried out as described in Methods Section.

rates was observed with both untreated cells of flocculent strains and hydrofluoric acid-treated organisms by treatment with 1,2 epoxypropane (5%, v/v) in 0.05M sodium acetate buffer (pH 4.5) overnight, complete deflocculation was not achieved (Table 16).

EFFECT OF MANNOSE ON THE SEDIMENTATION RATE OF UNTREATED AND HYDROFLUORIC ACID-TREATED *SACCHAROMYCES CEREVISIAE*

Mannose and other fermentable sugars deflocculate flocculent strains of brewer's yeast when included in the suspending medium (Eddy, 1955a). The effect of mannose on the sedimentation rate of untreated and hydrofluoric acid-treated *Saccharomyces cerevisiae* was studied. The sedimentation rates were measured using 0.05M sodium acetate buffer (pH 4.5) containing $CaCl_2$ (0.1%, w/v) and different concentrations of mannose.

The sedimentation rate of untreated flocculent *Saccharomyces cerevisiae* was decreased to a very low value characteristic of non-flocculent strains by mannose at a concentration of approximately 220 mmol per litre (Fig 26). However, mannose up to a concentration of approximately 330 mmol per litre did not have an appreciable effect on the sedimentation rate of hydrofluoric acid-treated organisms. (Fig. 26).

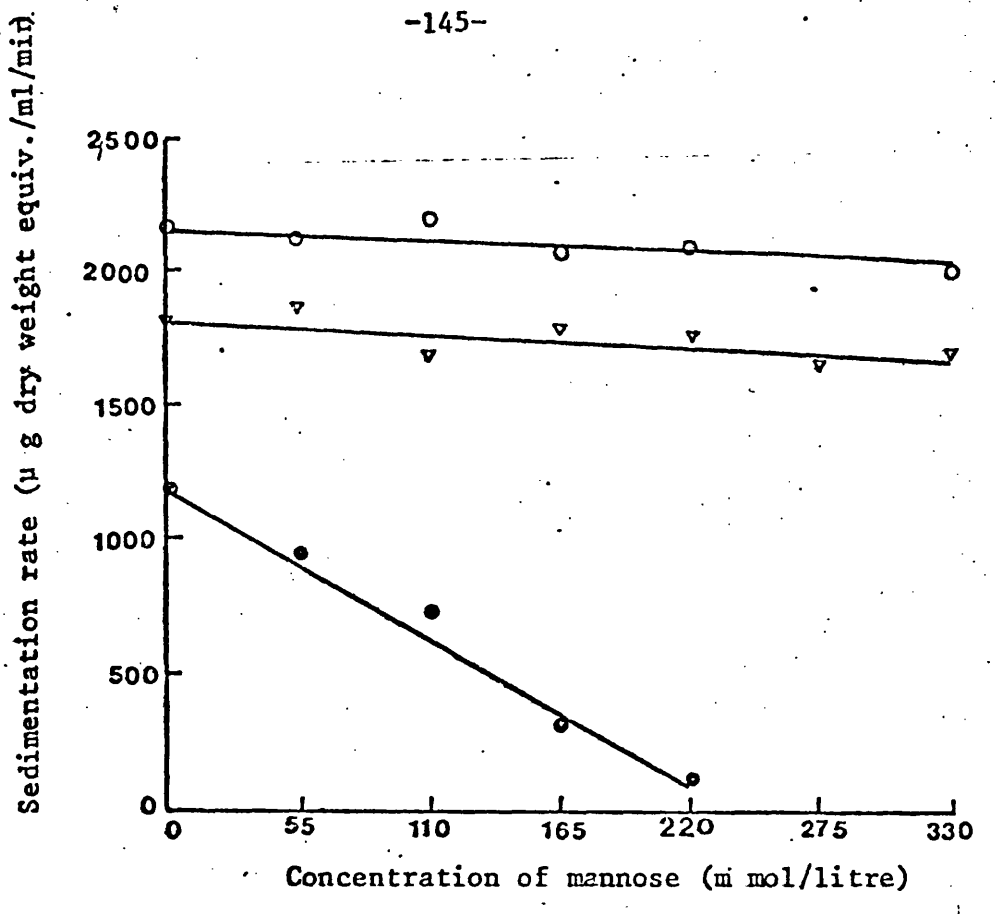


Figure 26. Effect of mannose on the sedimentation rate untreated and hydrofluoric acid-treated *Saccharomyces cerevisiae*. Sedimentation rates were measured in 0.05 M sodium acetate buffer (pH 4.5) containing $CaCl_2$ (0.1%, w/v) and different concentrations of mannose. ●——● indicates untreated cells of NCYC 1063, O——O HF-treated cells of NCYC 1063, Δ——Δ HF-treated cells of NCYC 366. Organisms were grown in MYGP medium and harvested in the stationary phase of growth. Values indicated are the means of four determinations. the 95% confidence limits were less than 8%.

EFFECT OF HYDROFLUORIC ACID TREATMENT ON SURFACE PROPERTIES OF *SACCHAROMYCES CEREVISIAE*

Various surface properties of whole cells before and after treatment with hydrofluoric acid (58 - 62%, v/v) were studied. These were: (a) binding of fluorescent antibody; (b) binding of fluorescent concanavalin A; and (c) electrophoretic mobility. All of these properties are believed to be associated with either the mannan or the mannan-protein of the yeast wall.

Hydrofluoric acid-treated *Saccharomyces cerevisiae* strains NCYC 366, NCYC 1004, NCYC 1005 and NCYC 1063 bound antibody raised against NCYC 366 much less avidly compared with untreated cells. However, both untreated and hydrofluoric acid-treated cells of all four strains bound fluorescent concanavalin A to the same extent. Hydrofluoric acid treatment had no effect on the capacity to bind fluorescent concanavalin A (Table 17).

The electrophoretic mobilities of *Saccharomyces cerevisiae* strains NCYC 366, NCYC 1004, NCYC 1005 and NCYC 1063 were measured before and after treatment with hydrofluoric acid in the pH range 2.0 - 8.0 using buffers of ionic strengths 0.05 and 0.005 (Figs. 27 - 30). As described earlier the mobility at pH 4.0 ($\mu_{4.0}$) gives a measure of the mobility due to phosphate groups of the phosphomannan whereas the change in the mobility between pH 3.0 and 7.0 ($\mu_{7.0} - \mu_{3.0}$) gives a measure of the contribution from protein (Eddy & Rudin, 1958b). Assuming a symmetrical decay of potential about the solid-liquid interface the electrophoretic mobility in buffers of ionic strength

Reagent	NCYC 366		NCYC 1004		NCYC 1005		NCYC 1063	
	Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated
Fluorescent antibody	+++++	+	+++++	+	+++++	+	+++++	+
Fluorescent concanavalin A	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++

Table 17 Binding of fluorescent antibody and fluorescent concanavalin A by untreated and hydrofluoric acid-treated strains of *Saccharomyces cerevisiae*

Key: +++++, bright fluorescence, +, faint fluorescence. Organisms were grown in MYGP medium and harvested in the stationary phase of growth.

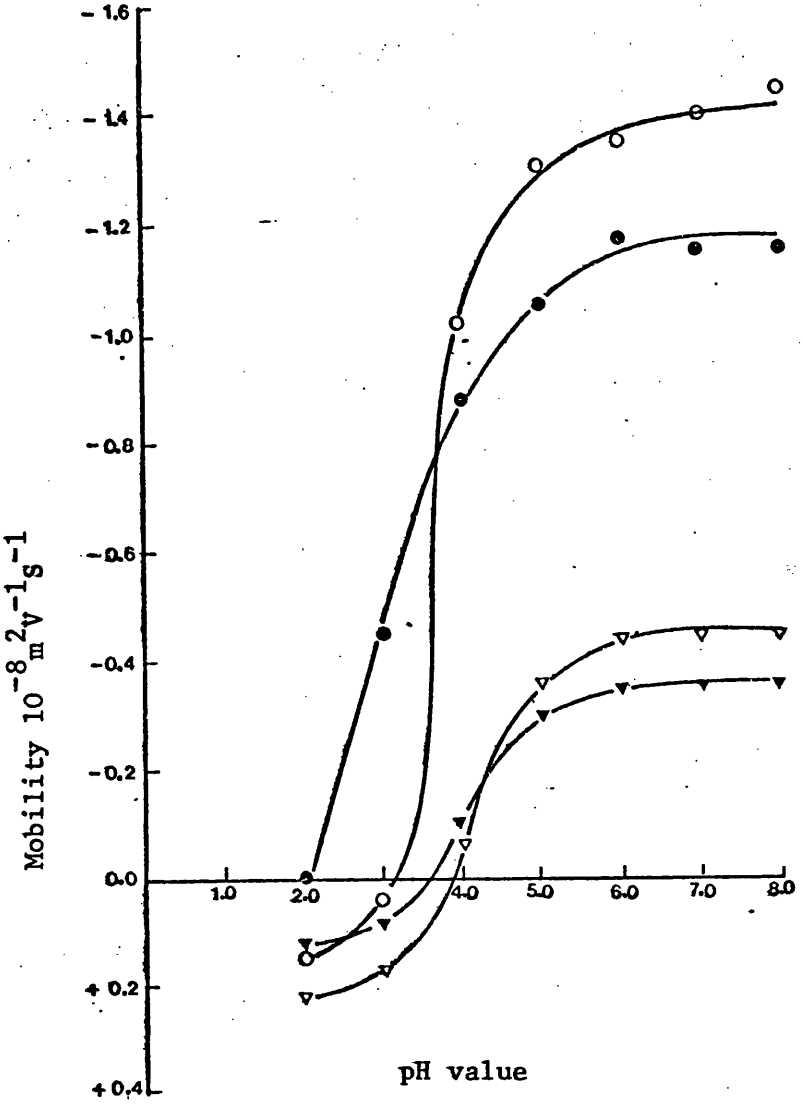


Figure 27 pH-Electrophoretic mobility curves of *Saccharomyces cerevisiae* NCYC 366, grown in MYGP medium (Wickerham, 1951) and harvested in the late stationary phase of growth. ●—● indicates untreated organisms (I = 0.05), O—O untreated organisms (I = 0.005), ▼—▼ HF-treated organisms (I = 0.05), ▽—▽ HF-treated organisms (I = 0.005).

Values indicated are the means of 20 observations. The standard error of the mean was less than 2%.

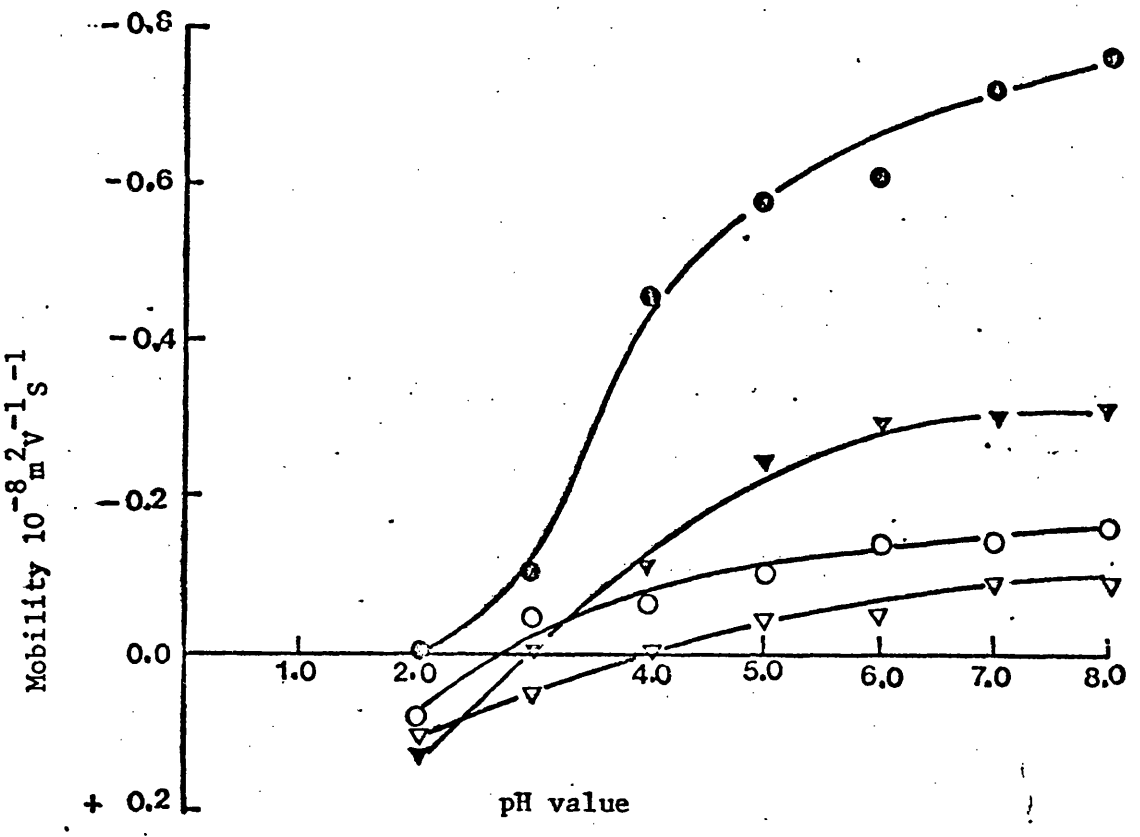


Figure 28 pH-Electrophoretic mobility curves of *Saccharomyces cerevisiae* NCYC 1004, grown in MYGP medium (Wickerham, 1951) and harvested in the late staticnary phase of growth. ●—● indicates untreated organisms (I = 0.05), O—O untreated organisms (I = 0.005), ▼—▼ HF-treated organisms (I = 0.05), ▽—▽ HF-treated organisms (I = 0.005).

Values indicated are the means of 20 observations. The standard error of the mean was less than 2%.

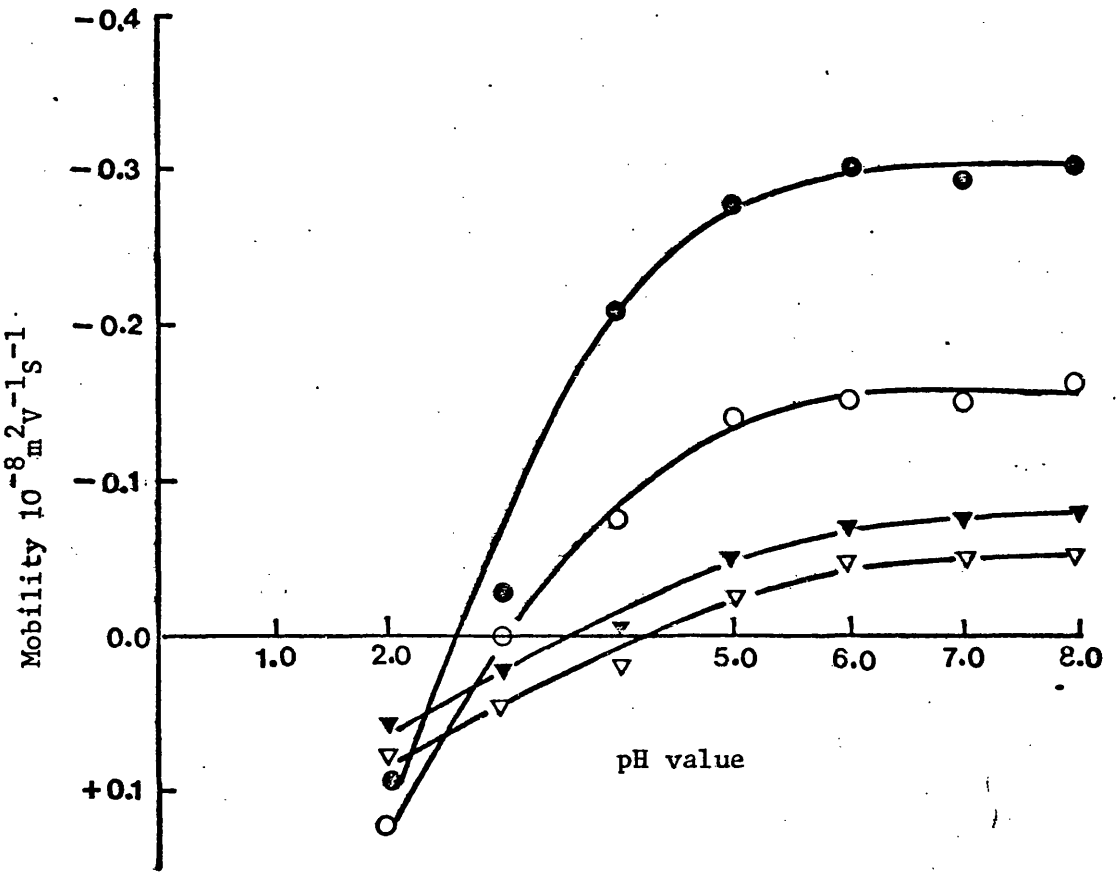


Figure 29 pH-Electrophoretic mobility curves of *Saccharomyces cerevisiae* NCYC 1005, grown in MYGP medium (Wickerham, 1951) and harvested in the late stationary phase of growth. ●—● indicates untreated organisms (I = 0.05), ○—○ untreated organisms (I = 0.005), ▼—▼ HF-treated organisms (I = 0.05), ▽—▽ HF-treated organisms (I = 0.005).
Values indicated are the means of 20 observations. The standard error of the mean was less than 2%.

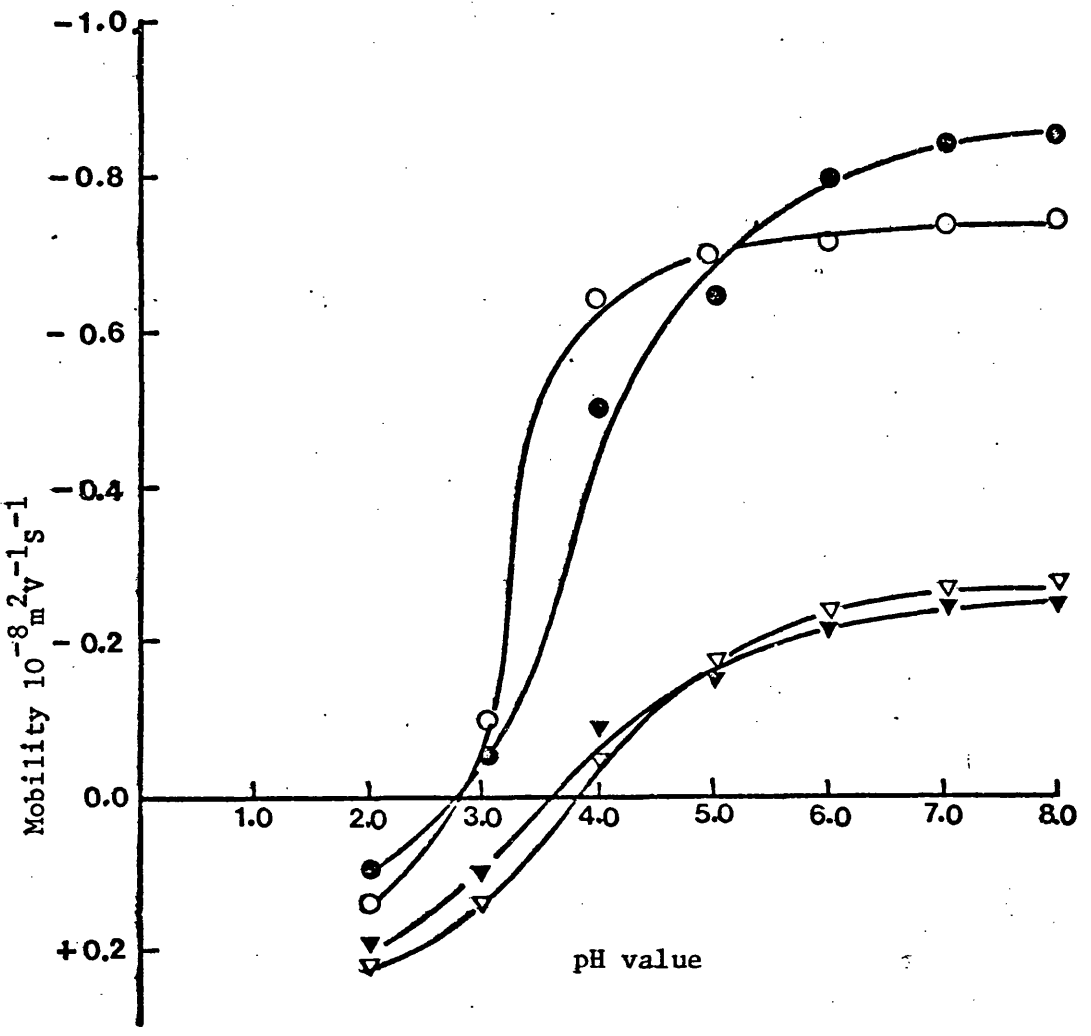


Figure 30 pH-Electrophoretic mobility curves of *Saccharomyces cerevisiae* NCYC 1063, grown in MYGP medium (Wickerham, 1951) and harvested in the late stationary phase of growth. ●—● indicates untreated organisms (I = 0.05), O—O untreated organisms (I = 0.005), ▼—▼ HF-treated organisms (I = 0.05), ▽—▽ HF-treated organisms (I = 0.005).

Values indicated are the means of 20 observations. The standard error of the mean was less than 2%.

0.05 and 0.005 depends on the surface layers of 1.4 and 4.2 nm of thickness, respectively (Fisher, 1975). Therefore this study involves a quantitative investigation of the three types of ionogenic groups found in the surface layers of the cell wall of 1.4 and 4.2 nm of thickness.

Untreated cells of NCYC 366 in buffers of ionic strength 0.05 showed a typical protein-phosphate type of pH-mobility curve without any positive mobility. The contribution by phosphate groups was greater than that of protein. Cells of the same strain in buffers of ionic strength 0.005 showed a higher contribution from both phosphate and protein, than at ionic strength 0.05. However the contrib ution from phosphate groups was lower than from protein. In addition, the positive mobility below pH 4.0 showed the participation of more amino groups at this lower ionic strength . The pH-mobility curves of NCYC 366 after hydrofluoric acid treatment showed a decrease in the overall mobility at both ionic strengths. Although these curves showed the participation of all three types of ionogenic groups, the contribution from phosphate groups was greatly decreased as compared with untreated cells.

The pH-mobility curve of untreated NCYC 1004 at ionic strength 0.05 indicated contributions from both phosphate and protein. Both NCYC 366 and NCYC 1004 which are non-flocculent strains did not show any positive mobility at this ionic strength. However, with NCYC 1004 the higher contribution to the overall

mobility came from protein . The overall negative mobility of NCYC 1004 at ionic strength 0.005 was unusually low. But as with NCYC 366 more amino groups were involved at this lower ionic strength, with protein contributing more than phosphate groups to the overall mobility. There was a considerable lowering of the overall negative mobility after treatment with hydrofluoric acid, at both ionic strengths. Here again the mobilities due to phosphate groups were decreased to a very great extent.

The overall negative mobilities of untreated cells of NCYC 1005 at both ionic strengths were much lower than those of the other three strains studied. Unlike non-flocculent strains (NCYC 366 and NCYC 1004) the flocculent strains (NCYC 1005 and NCYC 1063) showed positive mobilities at lower pH values at both ionic strengths. For untreated NCYC 1005, the contribution from protein was greater than from phosphate groups at both ionic strengths. The hydrofluoric acid-treated cells of NCYC 1005 showed typical amino-carboxyl type pH-mobility curves with virtually no participation from phosphate groups.

The pH-mobility curves of untreated NCYC 1063 at both ionic strengths indicated contributions from all three types of ionogenic groups, with the contribution from protein greater than from phosphate groups. Treatment with hydrofluoric acid was found to lower the mobility at pH 4.0 which is due to phosphate groups to a great extent as observed with other strains.

The decrease in mobilities due to phosphate groups ($\mu_{4.0}$) at both ionic strengths, after treatment with hydrofluoric acid

(58 - 62%, v/v), are given in Table 18. These results show that the hydrofluoric acid treatment removed 70 - 100% of the mobility due to phosphate groups. As a result the ratio protein mobility: phosphate mobility given by $(\mu_{7.0} - \mu_{3.0})/\mu_{4.0}$ increased from below 2.0 for untreated cells to above 3.5 and reaching values as high as 20.5 for hydrofluoric acid-treated cells (Table 19). In some strains, contributions from phosphate groups were completely removed and the negative mobility was due entirely to carboxyl groups.

EFFECT OF HYDROFLUORIC ACID TREATMENT ON CALCIUM BINDING BY CELL WALLS OF *SACCHAROMYCES CEREVISIAE*

Binding of calcium by untreated and hydrofluoric acid-treated walls of strains of *Saccharomyces cerevisiae* was studied by suspending 10 mM EDTA-washed cell walls at a concentration of 5 mg dry weight per ml in 0.05M sodium acetate buffer (pH 4.5) containing 3.6 mmol $CaCl_2$ and approximately 0.4 mCi of $Ca^{45}Cl_2$ per litre. The suspension was incubated at 30°C with stirring for 40 min.

Untreated walls bound approximately 300 - 400 µg calcium per 100 mg dry weight of walls. Hydrofluoric acid-treated walls bound much greater amounts of calcium ranging from 475 - 550 µ g calcium per 100mg dry weight walls (Table 20). There was a possibility that higher values obtained for calcium binding by hydrofluoric acid-treated walls were partly due to F^- ions retained in the walls following hydrofluoric acid treatment.

Strain	$\mu_{4.0}$ at I-0.05		% loss in mobility $\mu_{4.0}$	$\mu_{4.0}$ at I-0.005		% loss in mobility $\mu_{4.0}$
	Untreated	HF treated		Untreated	HF treated	
NCYC 366	-0.87	-0.10	88.5	-1.02	-0.06	94.1
NCYC 1004	-0.44	-0.13	70.5	-0.08	0.0	100
NCYC 1005	-0.21	-0.015	92.9	-0.085	+0.01	100
NCYC 1063	-0.45	-0.06	86.7	-0.63	-0.02	96.8

Table 18. Decrease in the mobilities due to phosphate groups ($\mu_{4.0}$) of strains of *Saccharomyces cerevisiae* after treatment with hydrofluoric acid (58 - 62%, v/v)

	Non-Flocculent				Flocculent			
	NCYC 366		NCYC 1004		NCYC 1005		NCYC 1063	
	Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated
$\mu_{7.0} - \mu_{3.0}/\mu_{4.0}$ at I - 0.05	0.83	4.30	1.39	3.00	1.10	entirely -COOH	1.78	5.67
$\mu_{7.0} - \mu_{3.0}/\mu_{4.0}$ at I - 0.005	1.34	10.33	1.38	entirely -COOH	1.80	entirely -COOH	1.08	20.5

Table 19. Ratio mobility protein: mobility phosphate ($\mu_{7.0} - \mu_{3.0}/\mu_{4.0}$) of strains of *Saccharomyces cerevisiae* before and after treatment with Hydrofluoric acid (58 - 62%, v/v). Organisms were grown in MYGP medium (Wickerham, 1951) and harvested in the stationary phase of growth.

	Yeast strain	Calcium bound μg/100 mg dry weight of walls		
		Untreated	HF-treated	KF-treated
Non-flocculent	NCYC 366	396 ± 11	531 ± 23	692 ± 31
	NCYC 1004	362 ± 13	487 ± 18	-
Flocculent	NCYC 1005	310 ± 09	550 ± 09	-
	NCYC 1063	394 ± 15	475 ± 23	688 ± 37

Table 20. Calcium binding by untreated and treated cell walls of strains of *Saccharomyces cerevisiae*. Values quoted are the means of four determinations ± 95% confidence limits. Organisms were grown in MYGP medium and harvested in the stationary phase of growth.

Therefore calcium binding by walls of one flocculent and one non-flocculent strain treated with potassium fluoride (11%, w/v), which had the same concentration of F^- ions as in the hydrofluoric acid treatment, was also studied.

Cell walls of NCYC 366 and NCYC 1063 were suspended in potassium fluoride solution (11%, w/v) at a concentration of 10 mg dry weight per ml for 3-5 h. Then they were washed five times with distilled water and freeze-dried. The calcium binding by these potassium fluoride-treated cell walls was studied as described earlier. Potassium fluoride-treated cell walls bound approximately 300 µg more calcium per 100 mg dry weight of walls as compared with untreated cell walls indicating the retention of F^- ions (Table 20).

DISCUSSION

PART I

POLYDIMETHYLSILOXANE (PDS) BINDING BY *SACCHAROMYCES CEREVISIAE*

It has been found by Vernon & Rose (1976) that *Saccharomyces cerevisiae* binds small amounts of PDS when suspended in a suitable buffer or grown in a medium containing the antifoam. Due to the insolubility of PDS in water, it has to be incorporated into aqueous media in the form of an emulsion. In their work, Vernon & Rose (1976) incorporated PDS into aqueous media by using Antifoam M-10 (Dow Corning Ltd., Barry, U.K.) which is a PDS-in-water emulsion. This emulsion contained a complex mixture of compounds, including emulsifiers and thickeners, in addition to PDS, and these compounds determine the chemical and physical properties of the emulsion to a very great extent. The presence of these ingredients in the emulsion was expected to have a significant effect on the binding of PDS by yeasts. Furthermore, emulsifiers and thickeners, being surface active themselves, would be expected to compete for binding sites on the yeast cell wall. Therefore it was thought appropriate to investigate the effect of these ingredients on PDS binding by *Saccharomyces cerevisiae*.

Antifoam M-10 contains two emulsifiers, namely polyoxyethylene sorbitan monostearate and glycerol monostearate and a thickener, sodium carboxymethyl cellulose. Emulsifiers basically are chemical combinations of two products, one having a strong affinity for water and the other a strong affinity for fat (oil). In the hydrophilic-lipophilic-balance (HLB) system of emulsifier classification, a number is assigned to each emulsifier which expresses the balance of the number and strength of its hydrophilic groups as compared with its lipophilic groups. The HLB scale

extends from zero to 20, and as the HLB value (i.e. the preponderance of hydrophilic groups) increases the emulsifiers become more soluble in water and their function changes from being water-in-oil emulsifiers to oil-in-water emulsifiers. While glycerol monostearate has a HLB value of 3.7, characteristic of lipophilic emulsifiers, polyoxyethylene sorbitan monostearate has a HLB value of 14.9 characteristic of hydrophilic emulsifiers. Each system to be emulsified has a so-called 'required HLB value' (optimum HLB value) which is determined experimentally and the emulsifiers or emulsifier blends possessing this HLB value should, in theory, be the most effective at promoting the formation and stability of the required emulsion. Emulsifier blends having the required HLB value are usually reckoned to be more effective in this respect than a single emulsifier having the required HLB value (Lecchini, 1971). Emulsions of the oil-in-water type have their best stability at HLB values around 11-12 (Boyde, Parkinson & Sherman, 1972). Antifoam M-10 which falls into this category has an HLB value of 10.4, and assuming this to be the required HLB value for such emulsions, Antifoam M-10 and similar PDS-in-water emulsions will have their best stability at this value.

In the present study a series of PDS-in-water emulsions with different concentrations of polyoxyethylene sorbitan monostearate or glycerol monostearate were used to study the saturation concentration of PDS bound by *Saccharomyces cerevisiae*. When the concentration of polyoxyethylene sorbitan monostearate or

glycerol monostearate was related to the saturation concentration of PDS bound by the organisms, any decrease in the concentration of either polyoxyethylene sorbitan monostearate or glycerol monostearate from their original value (i.e. the concentration in Antifoam M-10) led to an increase in the saturation concentration of PDS bound in a linear manner. This could be explained either of two ways. Firstly a situation may be envisaged where there is a competition between PDS and other surface-active ingredients, in the emulsion, for the binding sites on the surface of the yeast. In such a situation lowering the concentration of any one of the emulsifiers would lead to less competition making more binding sites available for PDS. Secondly, it is well known that polymeric material in solution are adsorbed more strongly from a poor solvent (less stable solution) than from a good solvent (more stable solution; Kipling, 1965). Adsorption from emulsions may be considered as an analogous situation where more adsorption could occur from a less stable emulsion than from a more stable emulsion. It was found that lowering the concentration of polyoxyethylene sorbitan monostearate or glycerol monostearate in the emulsion caused the HLB value to deviate from the required value which is 10.4, thereby causing a decrease in the stability. Therefore, on the basis of this supposed analogy, more adsorption of PDS could be expected from less stable emulsions than from more stable emulsions.

The other emulsions used in these experiments contained different amounts of sodium carboxymethylcellulose which is an ionic cellulose ether. Cellulose ethers in general are used in emulsions to achieve better binding and thickening. A decrease

in the concentration of sodium carboxylmethyl cellulose in the emulsions led to a decrease in the saturation concentration of PDS bound by *Saccharomyces cerevisiae*, from the emulsions, in a linear manner. Although a satisfactory explanation for this cannot be forwarded, it is possible that sodium carboxymethyl cellulose plays an accessory role in PDS binding by these organisms where it may help to bring about bonding between the hydrophobic PDS molecules and the hydrophilic surface of the yeast.

There is evidence from the work of Vernon & Rose (1976) which indicates that it is the wall of *Saccharomyces cerevisiae* that is responsible for binding PDS. This includes evidence from studies made on PDS binding by isolated walls and release of PDS on formation of sphaeroplasts from PDS-saturated organisms. The wall of *Saccharomyces cerevisiae* is a heterogeneous and highly complex organelle composed mainly of β-glucan, α-mannan and protein and a little chitin and lipid. It was therefore considered necessary to establish the nature of the components of the wall that are responsible for binding PDS. In order to establish which wall components of *Saccharomyces cerevisiae* NCYC 366 are responsible for binding PDS, intact organisms were treated with reagents capable of removing specific components from the wall, and the effect of the treatments on PDS binding studied. The reagents used in this study were trypsin, β(1-3) glucanase, potassium hydroxide (6%, w/v) and hydrofluoric acid (58 - 62%, v/v).

In the present study treatment of intact *Saccharomyces cerevisiae* NCYC 366 with trypsin or potassium hydroxide for different periods of time prior to studying PDS binding caused a very considerable decrease (40 - 60%) in the saturation concentration of PDS bound by the organisms.. The decrease was greater with potassium hydroxide - treated organisms than with trypsin-treated organisms. Investigations made on the composition of isolated walls before and after treatment with trypsin or potassium hydroxide indicated that both of these treatments led to the loss of most of the mannan, protein and phosphorus from the wall confirming the earlier results of Eddy (1958) and Lyons & Hough (1970 a, b, 1971), and McMurrough & Rose (1967) respectively. Therefore these results indicate that the binding sites for PDS on the wall of *Saccharomyces cerevisiae* are mostly associated with the phosphomannan-protein complex which is thought to be located in the outer layers of the cell wall by Lyons & Hough (1970b).

Treatment of intact *Saccharomyces cerevisiae* NCYC 366 with β(1-3) glucanase for short periods of time affected PDS binding by the organisms differently from that of trypsin or potassium hydroxide treatments. Intact organisms treated with β(1-3) glucanase bound much more PDS than untreated organisms. The analytical data on the composition of walls before and after treatment with β-glucanase showed that this treatment removed considerable amounts of glucan with smaller amounts of mannan, protein and phosphorus. The glucan components of the yeast wall are mainly responsible for the shape and rigidity of the cell. The major component of *Saccharomyces cerevisiae* glucan is a β(1-3) polymer

(Manners *et al.*, 1973a). Basidiomycete β(1-3) glucanse, as the name implies, catalyses hydrolysis of β(1-3) linkages of glucan. Therefore disruption of the glucan layer by this treatment could be expected to lead to the disorganisation of the outer layers of the wall as well. Treatment of intact organisms with β-glucanase could therefore lead to the exposure of new binding sites for PDS which are probably in the phosphomannan-protein inner in the surface layers.

Studies on PDS-release from saturated organisms following treatment with trypsin, β(1-3) glucanase or potassium hydroxide confirmed the results of the previous experiment. While trypsin and potassium hydroxide were very effective in releasing PDS from PDS-saturated organisms, β(1-3) glucanse was less effective. Treatment with potassium hydroxide released about 85% of the PDS while trypsin was equally effective in releasing PDS up to 81%. However, treatment with β(1-3) glucanase was able to release only about 55% of the PDS originally bound. Release of PDS by trypsin or potassium hydroxide again suggests that the binding sites for PDS reside in the phosphomannan-protein component of the cell wall. The release of about 55% of the original PDS content by β(1-3) glucanase may be due to removal of some phosphomannan-protein when the glucan framework was disrupted by this enzyme treatment. Therefore the evidence from PDS binding by treated organisms and PDS release from saturated organisms following treatment with trypsin, potassium hydroxide or β(1-3) glucanase suggests that the sites for binding PDS by *Saccharomyces cerevisiae* are located mainly if not solely in the phosphomannan-protein component of the cell wall.

The phosphomannan-protein complex carries three types of ionogenic groups, namely the phosphate groups in the mannan and carboxyl groups of the acidic amino-acid residues and amino groups of the basic amino-acid residues in the protein part of the complex. Due to the possibility that these groups participate in binding PDS to the yeast, it was necessary to assess the relative contributions from them. A modification of methods already used to excize phosphodiester groups involving treatment with hydrofluoric acid, was used in the present study to treat intact *Saccharomyces cerevisiae* NCYC 366 prior to studying PDS binding. Analytical studies made on isolated walls before and after this treatment showed that it led to the loss of about 90% of the wall phosphorus together with small amounts of glucan, mannan and protein. Experiments on PDS binding carried out using intact organisms that had been treated with hydrofluoric acid showed a decrease of about 50% in the saturation concentration of PDS bound by the organisms. This indicates that the phosphate groups of the phosphomannan are responsible for a significant percentage of binding sites for PDS, and that other ionogenic or non-ionogenic sites on the wall must also be involved.

Investigations on the nature of the surface of *Saccharomyces cerevisiae* NCYC 366 before and after treatment with trypsin, potassium hydroxide, β(1-3) glucanase or hydrofluoric acid revealed considerable changes following these treatments. The properties of the wall investigated were the surface charge and binding of fluorescein-labelled antibody and fluorescein-labelled concanavalin A. Electrophoretic mobility patterns which gave a measure of surface charge of *Saccharomyces cerevisiae* reveal quantitative information

about the ionogenic groups of the surface. The electrophoretic mobility of trypsin-treated organisms showed a decrease of about 20% in the mobility due to phosphate groups whereas the decrease in the mobility due to protein was much greater. Potassium hydroxide treatment lowered the mobility due to phosphate groups to a greater extent than trypsin. However, this treatment appears to have left a surface layer rich in protein giving a high mobility as expressed by the $\mu_{7.0} - \mu_{3.0}$ value. Hydrofluoric acid treatment lowered the mobility due to phosphate groups to a very low value whereas the decrease in the mobility due to protein was comparatively small. From these results it is apparent that the decrease in PDS binding observed due to these treatments could be ascribed to loss of ionogenic groups from the surface. Studies on trypsin digestion showed that the loss of large amounts of ionogenic groups from the protein together with smaller amount of phosphate groups from the surface caused a decrease of about 50% in the PDS binding ability of the wall. However potassium hydroxide treatment, which lowered the mobility due to phosphate groups of the surface to a greater extent than trypsin treatment but failed to remove the surface protein, brought about a greater decrease in the PDS binding ability of the wall. Therefore these results indicate that in addition to phosphate groups, ionogenic groups of the protein are involved in PDS binding. However phosphate groups would appear to make the main contribution. This conclusion is supported by the fact that the amounts of PDS bound by organisms treated with trypsin for different periods of time were directly proportional to the

residual phosphorus and mannan contents of the cell wall. The results obtained for hydrofluoric acid-treated organisms also are consistent with such an explanation. The organisms treated with β(1-3) glucanase showed an increase in the mobilities due to both phosphate groups and protein indicating an increased exposure of ionogenic groups. The greater PDS binding shown by organisms treated with β(1-3) glucanase too is therefore in agreement with the given explanation.

All known immunogenic groups of *Saccharomyces cerevisiae* are carried by the phosphomannan of the cell wall. Therefore the decreased binding of fluorescein-labelled antibody shown by trypsin - and potassium hydroxide-treated organisms confirmed the loss of phosphomannan from the wall. The decrease in the fluorescein-labelled antibody binding by hydrofluoric acid-treated organisms may be due to the loss of α-D-mannopyranosyl phosphate units which are important immunogenically in *Saccharomyces cerevisiae* (Raschke & Ballou, 1971), although the bulk of α-linkages in the mannan molecule appears to remain intact.

Studies on binding of fluorescein-labelled concanavalin A which specifically binds to α-mannan of the wall of *Saccharomyces cerevisiae* (Tkacz *et al.*, 1971) gave further evidence for loss of mannan from the wall following trypsin and potassium hydroxide treatments. Hydrofluoric acid -treated organisms bound fluorescein-labelled concanavalin A at the same intensity as compared with untreated organisms. This shows that the bulk of α-linkages in the

mannan chain are not affected by this treatment. Organisms treated with β(1-3)glucanase bound both fluorescein-labelled antibody and fluorescein-labelled concanavalin A with an intensity close to untreated organisms.

The evidence presented so far therefore indicates that ionogenic groups carried by phosphomannan-protein are responsible for binding PDS to the wall of *Saccharomyces cerevisiae* NCYC 366. Although both phosphate groups and ionogenic groups of the protein are implicated, phosphate groups appear to contribute more binding sites than ionogenic groups of the protein. This suggestion is also supported by the decreased PDS binding shown by concanavalin A-treated organisms. This decrease may be due to the concanavalin A, which reacts with α-mannan of the cell wall, rendering the phosphate groups inaccessible to PDS. Furthermore, organisms grown in MYGP medium (Wickerham, 1951) which had more phosphorus in the wall, bound more PDS than organisms grown in defined medium, in agreement with the above mentioned explanation.

Experiments carried out on the effect of surface charge on the PDS binding by *Saccharomyces cerevisiae* NCYC 366 indicated that the overall surface charge has no significant effect. This may be due to charged groups of the phosphate type which are believed to have a pK value lower than 2 being capable of contributing binding sites for PDS even at very low pH values at which the overall surface charge is very low. On the other hand, this may indicate that although the ionogenic groups of the cell wall are involved the binding itself is not of the ionic type. Therefore

the degree of dissociation of these groups has no effect on the binding of PDS.

Experiments carried out on the effect of PDS binding on the cell-wall properties of *Saccharomyces cerevisiae* NCYC 366 again s suggested the involvement of groups carried by the phosphomannan-protein of the wall. The electrophoretic mobility due to phosphodiester groups ($\mu_{4.0}$)of PDS-saturated organisms showed a decrease of about 11% as compared to that of untreated organisms. The relatively small mobility due to protein ($\mu_{7.0} - \mu_{3.0}$)of untreated organisms could not be detected in PDS-treated organisms. Furthermore PDS-saturated organisms showed less binding of both fluorescein-labelled antibody and fluorescein-labelled concanavalin A. These data again indicate that the ionogenic groups of phosphomannan-protein are the major contributory groups in binding PDS to the wall of *Saccharomyces cerevisiae* NCYC 366.

The droplet size in the antifoam emulsion also affected the amount of PDS bound by the yeast. When Antifoam M-10 emulsions with large droplet size were used, slightly more PDS was bound by the yeast. This fact again may be related to the stability of the emulsion. It is also not known whether or not PDS is directly bound to the yeast. It is possible that ingredients such as emulsifiers and the thickener in Antifoam M-10 play a direct role in binding PDS to the yeast. Such a suggestion seems plausible since emulsifiers and thickeners which are both oil and water compatible would be ideal in establishing bonding between the hydrophobic PDS and the hydrophilic cell wall. However a simple

mechanism for bonding between ionic groups of the emulsifiers or the thickener and ionic groups in the cell wall is not easily put forward. The notion that emulsifiers are involved in binding PDS to the cell wall appears to contradict the earlier suggestion of a competition between PDS and emulsifiers for the same binding sites on the wall. However it may be assumed that, after emulsification there is still an appreciable amount of free emulsifier in the preparation. It is possible that emulsifier molecules adsorbed on PDS droplets facilitate bonding between PDS and the wall, while free emulsifier molecules compete for the same binding sites on the wall.

Walls of strains of *Saccharomyces cerevisiae* are capable of binding, in addition to PDS, a host of other compounds such as hop constituents and proteins as well as bivalent cations such as calcium. Whereas the glucan component of the wall is mainly responsible for the shape and rigidity of the cell, phosphomannan-protein appears to be responsible for the various binding properties of the cell wall. Furthermore components responsible for intercellular binding of *Saccharomyces cerevisiae*, illustrated by sexual agglutination and flocculation, also are associated with the phosphomannan-protein of the cell wall.

The invertase activity of *Saccharomyces cerevisiae* is associated with a glycoprotein in the wall. A highly purified invertase prepared by Neumannn & Lampen (1967) contained about 50% mannan and 3% glucosamine. The protein part of the molecule contained high proportions of aspartic acid, serine and threonine. Investigations

made on the invertase activity of PDS-saturated *Saccharomyces cerevisiae* NCYC 366 showed that binding of PDS had no obvious effect on the invertase activity of the organisms. This might be considered as a very important advantage in the use of PDS-antifoams in brewery fermentations.

PART II

ROLE OF PHOSPHODIESTER-LINKED MANNAN IN FLOCCULATION OF *SACCHAROMYCES CEREVISIAE*

Involvement of phosphate groups of the cell-wall phosphomannan and carboxyl groups of the acidic amino-acid residues of the cell-wall protein in flocculation of *Saccharomyces cerevisiae* has been claimed by many workers. Harris (1959), Mill (1964b) and Stewart *et al.* (1975) believe that carboxyl groups are responsible for the formation of cross bridges through Ca^{2+} ions which are involved in flocculation. However Lyons & Hough (1970 a, b, 1971) believe that it is the phosphate groups and not the carboxyl groups that are involved in the formation of cross bridges through Ca^{2+} ions. The object of the work on flocculation of *Saccharomyces cerevisiae* reported in this thesis was to obtain information which might allow an assessment to be made of the relative contributions of acidic amino-acid residues and phosphodiester linkages in flocculation. To obtain this information a technique was used which specifically excises the phosphodiester linkages from the cell-wall mannan.

The analytical data on isolated walls of *Saccharomyces cerevisiae* before and after treatment with hydrofluoric acid indicated that this treatment led to the excision of most of the phosphodiester linkages. While this treatment extracted about 80 - 90% of the cell-wall phosphorus, the losses in the other components of the cell wall were very low and probably within the limits of error of the analytical methods. Treatment of phosphomannan extracted from yeast cell walls with hydrofluoric acid (40%, v/v) was reported to remove only about 70% of the

phosphorus by Cawley *et al.* (1972). The use of more concentrated hydrofluoric acid (58 - 62%, v/v) appears to be more efficient in removing phosphorus from phosphomannan in the intact wall. However the treatment of isolated walls with hydrofluoric acid (58 - 62%, v/v) failed to remove phosphorus completely from the walls. This may be due to the fact that the hydrofluoric acid was unable to penetrate deep enough to extract phosphorus from certain inner layers. Staining with fluorescein-labelled concanavalin A, which binds specifically to the α-mannan of the cell wall (Tkacz *et al.*, 1971) was not affected by the pretreatment of *Saccharomyces cerevisiae* with hydrofluoric acid. This shows that the bulk of the α-linkages in the mannan was not affected by this treatment. However hydrofluoric acid-treated organisms showed decreased binding of fluorescein-labelled antibody raised against NCYC 366. This may be due to the loss of α-D-mannopyranosyl phosphate units which are important immunogenically in *Saccharomyces cerevisiae* (Raschke & Ballou, 1971).

Studies made on sedimentation rates of untreated cells of strains of *Saccharomyces cerevisiae* showed that there is no correlation between the cell-wall phosphorus contents and their sedimentation rates. Lyons & Hough (1970b) who studied the phosphorus contents of different fractions obtained from walls of various strains of *Saccharomyces cerevisiae* observed that fractions from the outer layers of the walls of flocculent strains had more phosphorus than those of the walls of non-flocculent strains. However in the present study electrophoretic mobility measurements at pH 4.0,

which gives a measure of the phosphate groups in the outer layers of the wall up to a depth of 4.2 nm (Eddy & Rudin, 1958b; Fisher, 1975), did not indicate such high contents of phosphorus in the outer layers of the cell wall of flocculent strains as compared with non-flocculent strains. The electrophoretic mobility patterns of untreated organisms did not show any trend which could be related to their flocculation behaviour. The mobility due to phosphate ($\mu_{4.0}$) and the mobility due to protein ($\mu_{7.0} - \mu_{3.0}$) varied from strain to strain but again no significant trend was noted which could be related to their sedimentation rates.

The increased sedimentation rates shown by hydrofluoric acid-treated organisms were rather surprising as the removal of phosphate groups from the cell-wall phosphomannan was expected at least not to increase the sedimentation rates. The hydrofluoric acid-treated organisms showed a very large decrease in the mobility due to phosphate groups ($\mu_{4.0}$) whereas the decrease in the mobility due to protein ($\mu_{7.0} - \mu_{3.0}$) was much smaller. There was no indication of an exposure of new ionogenic groups following hydrofluoric acid-treatment. In some strains, any mobility due to phosphate ($\mu_{4.0}$) after hydrofluoric acid treatment was completely absent. As a consequence, the ratio mobility protein: mobility phosphate increased to a very high value as compared with untreated cells. These findings are consistent with some earlier reports on flocculation by Masschelein *et al.* (1963), Jeunehomme - Ramos *et al.* (1964) and

Kijima (1964). Masschelein *et al.* (1963) stated that it is essentially the ratio of mannan to protein in the wall that determines the flocculation behaviour of yeasts, and that flocculent strains possessed an intracellular mechanism which allowed rapid utilization of mannan in the stationary phase of growth. Kijima (1964) who obtained two materials, flocculent and non-flocculent, by treatment of yeast with dilute alkali, found that the flocculent material had a high protein: polysaccharide ratio compared with the non-flocculent material. These findings imply that it is the ratio protein: polysaccharide (mannan) in the cell wall which determines the flocculent or non-flocculent behaviour of yeasts. It may well be that the groups actually involved are the carboxyl groups of the wall protein and the phosphate groups of the mannan.

Another interesting property of the hydrofluoric acid-treated organisms was the very low overall charge carried by them. This finding is in agreement with the observations made earlier by Jansen & Mendlik (1951). By electrophoretic mobility measurments of flocculent and non-flocculent strains of *Saccharomyces cerevisiae*, Jansen & Mendlik (1951) reported that, although all strains carried a negative charge at pH values of beer and wort (pH 3.8 - 5.6), the flocculent strains carry a lower charge than non-flocculent strains. Although it is now known that this is not true for all strains this may be a contributing factor which determines the flocculent character in some strains such as NCYC 1005 which carries a very low surface charge. The very low surface charge carried by hydrofluoric acid-treated

organisms also may be a contributing factor to the increased sedimentation rates shown by them.

There was a possibility that these increased sedimentation rates observed following hydrofluoric acid treatment of organisms could be due to retention of F^- ions or due to the action of hydrofluoric acid on phospholipids (Shaw & Stead, 1974). However treatment of non-flocculent strains with potassium fluoride (11%, w/v), which gave the same F^- ion concentration in the suspension as with hydrofluoric acid treatment, failed to cause flocculation of these strains. Also the extraction of lipid from intact organisms failed to cause any increase in the sedimentation rates of flocculent or non-flocculent strains. Therefore the retention of F^- ions in the envelope layers or the loss of phospholipids following hydrofluoric acid treatment, do not appear to be responsible for the increased sedimentation rates observed following this treatment. The decrease in the sedimentation rates of flocculent strains following lipid extraction may be due to loss of other hydrophobic cell-wall components (possibly proteins) due to this treatment.

Both untreated flocculent strains and hydrofluoric acid-treated organisms reacted similarly to most of the secondary treatments to which they were submitted, indicating that a similar mechanism of flocculation operates in both instances. Both untreated flocculent strains and hydrofluoric acid-treated organisms were rendered non-flocculent by washing in either deionized water or 10 mM EDTA. Addition of small

concentrations of Ca^{2+} ions (about 0.1%, w/v $CaCl_2$) to the suspension caused washed cells to reflocculate. These findings show that Ca^{2+} ions are an essential requirement for the expression of flocculence either inherent or induced by hydrofluoric acid treatment. The need to wash hydrofluoric acid-treated organisms with 10 mM EDTA instead of deionized water to deflocculate them may indicate that Ca^{2+} ions are more tightly bound by these treated organisms, as observed with some highly flocculent strains of *Saccharomyces cerevisiae* by Stewart *et al.* (1975).

The hypothesis which explains flocculation in terms of cross-bridge formation through Ca^{2+} ions linking carboxyl groups on adjacent yeast cells was put forward by Harris (1959) and was later confirmed by Mill (1964b) and Stewart *et al.* (1975) These carboxyl groups are carried in the acidic amino-acid residues of the cell-wall protein. Many workers have used 1,2 epoxypropane to esterify carboxyl groups found in the surface layers of micro-organisms (Gitten & James, 1963; Mill, 1964b; Lyons & Hough, 1970b). In the present study esterification of carboxyl groups of untreated flocculent strains and hydrofluoric acid-treated organisms with 1,2 epoxypropane led to a very considerable decrease in the sedimentation rates. This treatment has been reported to deflocculate potentially flocculent strains of *Saccharomyces cerevisiae* by Mill (1964b). A decrease of about 20% in the calcium binding capacity of walls of *Saccharomyces cerevisiae* following the same treatment was observed by Lyons & Hough (1970b). These effects indicate the

involvement of carboxyl groups in flocculation and calcium binding. On the same basis the observed effects following esterification of carboxyl groups on sedimentation rates of both untreated flocculent strains and hydrofluoric acid-treated organisms indicate the involvement of these groups in flocculation. However it is believed at least by some workers (Gittens & James, 1963) that treatment of micro-organisms with 1,2 epoxypropane does not cause complete esterification of the carboxyl groups in the surface layers of the wall. Therefore, although the involvement of carboxyl groups in flocculation is indicated by my results, the degree of involvement could not be accurately assessed. The participation of carboxyl groups in flocculation may be much greater than indicated by the observed decrease in the sedimentation rates following 1,2 epoxypropane treatment. These findings indicate that, in both untreated flocculent strains and hydrofluoric acid-treated organisms, a common mechanism involving Ca^{2+} ions and most probably carboxyl groups operates.

Fermentable sugars have been reported to deflocculate flocculent strains of brewer's yeast when included in the suspending medium (Eddy 1955a). The most effective were mannose and maltose. Mannose at a concentration of approximately 100 -200 mM was sufficient to deflocculate most strains of flocculent yeasts. In the present study mannose at a concentration of about 220 mM was sufficient to deflocculate untreated *Saccharomyces cerevisiae* NCYC 1063 which is a flocculent strain. However, when the effect of mannose on the sedimentation rate of hydrofluoric acid-treated

organisms was tested no appreciable effect was noted even at concentrations as high as 330 mM. Although the mechanism of deflocculation of flocculent yeasts by fermentable sugars is not adequately explained, it is believed that the sugars act selectively at different sites on the cell surface (Eddy, 1955a). The inability of mannose to deflocculate hydrofluoric acid-treated organisms may be due to the loss of reactive sites from the wall following hydrofluoric acid treatment. These reactive sites may well be associated with the phosphodiester linkages of mannan and the mannose or mannobiose residues which lie distal to the phosphodiester linkages and are lost during hydrofluoric acid treatment.

Experiments on calcium binding by untreated walls of *Saccharomyces cerevisiae* showed that the amount of calcium bound varies from strain to strain. However a trend could not be found in the amount of calcium bound by the walls of these four strains to the flocculent or non-flocculent behaviour shown by them. Lyons & Hough (1970b) have reported that esterification of surface carboxyl groups with 1,2 epoxypropane caused a decrease of only 20% in the calcium binding capacity of the walls. On the basis of this finding, they concluded that the part played by carboxyl groups in calcium binding is minor compared to that of phosphate groups. However, treatment of micro-organisms with 1,2 epoxypropane has been reported to cause incomplete esterification of surface carboxyl groups (Gitten & James, 1963). Therefore an assessment of the degree of involvement of carboxyl

groups in calcium binding cannot be obtained by this method. The higher values obtained for calcium binding by hydrofluoric acid-treated walls of strains of *Saccharomyces cerevisiae* would appear to be partly due to retention of F^- ions following the hydrofluoric acid treatment. From calcium-binding studies on potassium fluoride-treated walls, the calculated amount of calcium bound by the walls due to retention of F^- ions is approximately 300 µg per 100 mg dry weight of walls. When this value is subtracted from the amount of calcium bound by hydrofluoric acid-treated walls the contribution to the calcium binding from wall components is approximately 175 - 250 µg per 100 mg dry weight of walls. From these values it appears that, after removal of most of the phosphorus, the cell wall is still capable of binding appreciable amounts of calcium. Therefore both phosphate and carboxyl groups appear to play an equally important part in calcium binding.

From data previously reported and those given in this thesis on calcium cross-bridging hypothesis of flocculation, it is postulated that while carboxyl groups of acidic proteins in the cell wall are directly involved in the formation of cross-bridges through Ca^{2+} ions the phosphate groups of the phosphomannan are not. These latter groups however create a repulsive force between adjacent yeast cells due to their high negative charge. The flocculent or non-flocculent character of strains of *Saccharomyces cerevisiae* depends on the relative magnitude of the attractive and repulsive forces due to these groups. When

there is sufficient cross-bridge formation between carboxyl groups to overcome the repulsive forces due to phosphate groups, flocculation would occur. Both carboxyl groups and phosphate groups appear to be responsible for binding Ca^{2+} ions. However the nature of the binding to the two types of groups may not be the same. Therefore it may well be that it is the stereospecific manner in which calcium is bound and not the absolute amount that is important to flocculation, as suggested recently by Stewart *et al.* (1975).

REFERENCES

Agrawal, B.B.L. & Goldstein, I.J. (1968). Can. J. Biochem. *46*, 1147.

Akin, C. & Krabbe, E. (1966). Proc. Amer. Soc. Brew. Chem. 166.

Alterthum, F. & Rose, A.H. (1973). J. Gen. Microbiol. *77*, 371.

Ammon, R. & Hinsberg, K. (1936). Z. Physiol. Chem. *239*, 207.

Anderson, F.B. (1967). Symposium on yeast protoplasts - Abhandlungun der Deutschen Akademie der Wissenschaften Zur Berlin, 61.

Archibald, A.R., Baddiley, J. & Shaukat, G.A. (1968). Biochem. J. *110*, 583.

Bacon, J.S.D., Milne, B.D., Taylor, I.F. & Webbley, D.M. (1965). Biochem. J. *95*, 28c.

Bacon, J.S.D., Davidson, E.D., Jones, D. & Taylor, I.F. (1966). Biochem. J. *101*, 36c.

Bacon, J.S.D. & Farmer, V.C. (1968). Biochem. J. *110*, 34p.

Bacon, J.S.D., Farmer, V.C., Jones, D. & Taylor, I.F. (1969). Biochem. J. *114*, 557.

Baker, E.H., Barry, A.T. & Hunter, M.J. (1946). Ind. Engng. Chem. *38*, 1117.

Baker, D.A. & Kirsop, B.H. (1972). J. Inst. Brew. *78*, 454.

Ballou, C.E. (1970). J. Biol. Chem. *245*, 1197.

Ballou, C.E. (1976). In "Adv. Microbial Physiol." (Rose, A.H. & Tempest, D.W.,ed) *14*, 93. (Academic Press, London).

Ballou, C.E. (unpublished) Cited by Ballou, C.E. (1976). In "Adv. Microbial Physiol." (Rose, A.H. & Tempest, D.W., ed) *14*, 93. (Academic Press, London).

Banks, W.H. (1957). Proc. Intern. Congr. Surface activity, 2nd London, *1*, 16.

Barry, V.C. & Dillon, T. (1943). Proc. R. Ir. Acad., Sect B *49*, 177.

Bartnicki-Garcia, S. (1968). A. Rev. Microbiol. *22*, 87.

Bass, R.L. (1959). Chem. and Ind. (London) 912.

Becze de, G.I. (1962). Wallerstein Labs. Commun. *25* , 43.

Bell, D.J. & Northcote, D.H. (1950). J. Chem. Soc. 1944.

Bernan, K. (1968). In "Adv. Microbial Physiol". (Rose, A.H. & Wilkinson, J.F. ed) *2*, 143 (Academic Press, London).

Bhute, R.S. (1971). J. Sci. and Ind. Res. *30*(5), 241.

Bianchi, D.E. (1967). Antonie Van Leeuwenhoek *33*, 324.

B.I.R.F. Annual Report (Biochemistry section, 1972). J. Inst. Brew. *78*, 129.

Boyde, J., Parkinson, C. & Sherman, P. (1972). J. Colloid Interface Sci. *41*(2), 359.

Bridgman, P.W. (1949). In "Physics of High Pressure" (Bell, London, 2nd edition).

Briley, M.S., Illingworth, R.F., Rose, A.H. & Fisher, D.J. (1970). J. Bacteriol. *104*, 588.

Brock, T.D. (1959). J. Bacteriol. *78*, 59.

Buecher Jr., E.J. (1968). PhD Thesis, University of California, Davis.

Bull, A.T. & Chesters, C.G.C. (1966). Adv. Enzymol. *28*, 325.

Burger, M.M. & Glaser, L. (1964). J. Biol. Chem. *239*, 3168.

Burns, J.A. (1937). J. Inst. Brew. *43*, 31.

Campbell, I. (1967). Proc. Euro. Brew. Conv., Madrid, 145.

Cawley, T.N. & Letters, R. (1968). Biochem. J. *110*, 9p.

Cawley, T.N. & Letters, R. (1969). Biochem. J. *115* 9p.

Cawley, T.N. & Ballou, C.E. (1972). J. Bacteriol. *111*, 690.

Cawley, T.N., Harrington, M.G. & Letters, R. (1972). Biochem. J. *129*, 711.

Chattaway, F.W. (1968). J. Gen. Microbiol. *51*, 367.

Chieko, K., Nunokawa, Y. & Akiyama, H. (1971). Rep. Res. Inst. Brew. *143*, 31.

Chen, P.S., Toribara, T.Y. & Warner, H. (1956). Anal. Chem. *28* 1756.

Chester, V.E. (1963). Proc. Roy. Soc. (B) *162*, 555.

Cifonelli, J.A. & Smith, F. (1955). J. Amer. Chem. Soc. *77*, 5682.

Clarke, A.E. & Stone, B.A. (1963). Rev. Pure. Appl. Chem. *13*, 134.

Clayton, E., Howard, G.A. & Martin, P.A. (1972). Proc. Amer. Soc. Brew. Chem. 78.

Colonna, W.J. & Lampen J.O. (1974a). Biochemistry. *13*, 2741.

Colonna, W.J. & Lampen, J.O. (1974b). Biochemistry. *13*, 2748.

Comrie, A.A.D. (1952). Wallerstein Labs. Commun. *15*, 339.

Cook, A.H. (1963). Proc. Euro. Brew. Conv., Brussels 477.

Cook, A.H. (1969). Proc. Euro. Brew. Conv., Interlaken 225.

Coppock, D. (1950). Br. Pat. 631,507.

Cowland, T.W. (1968). J. Inst. Brew. *74*, 457.

Crandall, M.A. & Brock, T.D. (1968a). Science *161*, 473.

Crandall, M.A. & Brock , T.D. (1968b). Nature *219*, 533.

Crandall, M.A. & Brock, T.D. (1968c). Bacteriol. Rev. *32*(3), 139.

Crandall, M.A. & Caulton, J.H. (1973). Exp. Cell. Res. *82*, 159.

Crandall, M.A., Lawrence, L.M. & Saunders, R.M. (1974). Proc. Nat. Acad. Sci., U.S.A. *71* (1) 26.

Currie, C.C. (1953). U.S. Pat. 2,632,736.

Day, A.W., Poon, N.H. & Stewart, G.G. (1975).. Can. J. Microbiol. *21*, 558.

Dische, Z. (1927). Biochem. Z. *189*, 77.

Dische, Z. (1930). Mikrochemie *8*, 4.

Dixon, I.J. (1967). J. Inst. Brew. *73*, 488.

Dixon, I.J. & Leach, A.A. (1968). J. Inst. Brew. *74*, 63.

Dumtze, W., MacKay, V. & Manney, T.R. (1970). Science *168*, 1472.

Dunwell, J.L., Ahmad, E. & Rose, A.H. (1961). Biochem.. Biophys. Acta. *51*, 604.

Eddy, A.A. (1955a). J. Inst. Brew. *61*, 313.

Eddy, A.A. (1955b). Proc. Euro. Brew. Conv., Baden-Baden 65.

Eddy, A.A. (1958). Proc. Roy. Soc. B. *149*, 425.

Eddy, A.A. & Rudin, A.D. (1958a). Proc. Roy. Soc. B *148*, 419.

Eddy, A.A. & Rudin, A.D. (1958b). J. Inst. Brew. *64*, 139.

Eddy, A.A. & Rudin, A.D. (1958c). J. Inst. Brew. *64*, 19.

Ellison, A.H. & Zisman, W.A. (1956). J. Phys. Chem. *60*, 416.

Emeis, C.C. (1970). Brewer's Digest *45* (7), 66.

Evans, J.I. & Hall, M.J. (1971). Proc. Biochem. *6* (4), 23.

Evans, J.I. (1972). Proc Biochem. (April), 29.

Falcone, G. & Nickerson, W.J. (1956). Science N.Y., *124* 272.

Fisher, D.J. (1975). J. Inst. Brew 81, 107.

Fraser, A. R. & Russell, J.D. (1969). Clay Minerals *8*, 229.

Geilenkotten, I. & Nyns, E.J. (1970a). Bull. Anc. Etud. Brasserie Louvain *66*(1), 19.

Geilenkotten, I. & Nyns, E.J. (1970b). Bull. Anc. Etud. Brasserie Louvain *66* (2), 59.

Geilenkotten, I. & Nyns, E.J. (1971). Brewer's Digest (April), 64.

Gilliland, R.B. (1951). Proc. Euro. Brew. Conv., Brighton 35.

Gilliland, R.B. (1955). Brewer's Guild J.*41*,246.

Gilliland, R.B. (1957). Wallerstein Labs. Commun. *20*, 41.

Gilliland, R.B. (1958). In "Recent Studies in Yeast and Their Significance in Industry" S.C.I. Monograph No. 3 103.

Gilliland, R.B. (1971a) In "Modern Brewing Technology" (Findley, W.P.K. ed) 108 MacMillan Press.

Gilliland, R.B. (1971b). Brew. Guard. *100*(10) 29.

Gilliland, R.B. (1971c). J. Inst. Brew. *77*,, 276.

Ginterova, A. & Janotkova, D. (1965). J. Inst. Brew. *71* 417.

Gittens, G.J. & James, A.M. (1963). Biochim. Biophys. Acta. *66* , 237.

Gorin, P.A.J. & Perlin, A.S. (1956). Can. J. Chem.*34* , 1796.

Gorin, P.A.J. & Spencer, J.F.T. (1968). Adv. Carb. Chem. *23*, 367.

Greenshields, R.N., Yates, J, Sharp, P. & Davies, T.C. M. (1972) J. Inst. Brew. *78*, 236.

Griffin, S.R. & MacWIlliam, I.C. (1969). J. Inst. Brew. *75*, 355.

Gurin, S. & Hood, D.B. (1939). J. Biol. Chem. *131*, 211.

Harris, J.O. (1959). J. Inst. Brew. *65*, 5.

Hartong, B.D. (1951). Proc. Euro. Brew. Conv., Brighton, 110.

Hasenclaver, H.F. & Mitchell, W.O. (1946). J. Immunology *93*, 763.

Hassid, W.Z., Joslyn, M.A. & McCready, R.M. (1941). J. Amer. Chem. Soc.*63* , 295.

Haworth, W.N., Hirst, E.L. & Isherwood, F.A. (1937). J. Chem. Soc., 784.

Haworth, W.N., Heath, R.L. & Peat, S. (1941). J. Chem. Soc., 833.

Helm, E., Nohr, B. & Thorne, R.S.W. (1953). Wallerstein Labs. Commun. *16*, 315.

Hennig, K. & Ay, H. (1938). Biochem. Z. *299* , 123.

Holzman, G., MacAllister, R.V. & Niemann, C . (1947). J. Biol. Chem. *171*, 27.

Hough, J.S. (1957). J. Inst. Brew. *63*, 483.

Houwink, A.L. & Kreger, D.R. (1953). Antonie van Leeuwenhoek *19* , 1.

Hughes, D.E., Wimpenny, J.W.T. & Lloyd, D. (1971). In "Methods in Microbiology" Vol 5B (Norris, J.R. & Ribbons, D.W. Ed) 1 (Academic Press, London).

Hunter, K. & Rose, A.H. (1972). Biochim. Biophys. Acta *260*, 639.

Hurd, C.B. (1946). J. Amer.Chem. Soc. *68*, 364.

Ito, U. (1967a). Memoirs Coll. Sci. Univ. Kyota A *31*(2), 137.

Ito, U. (1967b). Memoirs Coll. Sci. Univ. Kyoto A *31*(2), 107.

Ito, U. (1967c). Memoirs Coll. Sci. Univ. Kyoto A *31*(2), 117.

Ito, U. (1967d). Memoirs Coll. Sci. Univ. Kyoto A *31*(2), 127.

Jansen, H.E. (1958). In "Chemistry and Biology of Yeasts" (Cook, A.H. ed) 635 (Academic Press Inc. New York).

Jansen, H.E. & Mendlik, F. (1951). Proc. Euro. Brew. Conv. Brighton 59.

Jansen, H.E. & Mendlik, F. (1953). Proc. Euro. Brew. Conv. Nice 143,

Jarvis, N.L. (1966). J. Phys. Chem. *70* , 10.

Jonas, V., Briess, R. & Minek, M.K. (1944). Schweiz. Brau. Rund. 55.

Jones, G.H. & Ballou, C.E. (1968). J. Bid. Chem. *243*, 2443.

Jeunehomme-Ramos, C., Castiau, M. & Masschelein, C.A. (1964). Rev. Ferm. Ind. Alim. *19*, 18.

Kato, S. & Nishikawa, N. (1957). Bull. Brew. Sci., Tokyo. *3*, 39.

Kessler, G. & Nickerson, W.J. (1959). J. Biol. Chem. *234*,2281.

Kijima, M. (1954). J. Inst. Brew.*60* , 223.

Kijima, M. (1964). Rep. Res. Lab., Kirin Brewing Co, Yokohama *7*, 7.

Kipling, J.J. (1965). In "Absorption from Solutions of Non-Electrolytes" 134 (Academic Press, London).

Klaushofer, H. & Dorfwirth, K. (1970). Mitteilungen der Versuchsanstalt fur Garungsgewerbe und des Instuts fiir Angewandte Mikrobiologie (Wien) *24* (2), 35.

Klaushofer, H., Mahr, H. & Szilvinyi, A. (1961). Mitt. Vers. Gar. Wien *15*, 10.

Kleyn, J. & Hough, J.S. (1971). Ann. Rev. Microbiol. *25*, 583.

Kocourek, J. & Ballou, C.E. (1969). J. Bacteriol. *100*, 1175.

Köffler, H. & Goldschmidt, M.C. (1950). Ind. Engng, Chem. *42*, 1819.

Kölbel, H. (1947). Z. Naturf. B. *2*, 382.

Korn, E.D. & Northcote, D.H. (1960). Biochem. J. *75*, 12.

Kristapsons, M. (1972). Khim-Farm. Zh. *6* (5), 27.

Lampen, J.O. (1968). Antonie Van Leeuwenhoek *34*, 1.

Lecchini, S. (1971). IFST Proceedings *4*(2), 50.

Lee, Y.C. & Ballou, C.E. (1965). Biochemistry NY *4* , 257.

Letters, R. (1968). In "Aspects of Yeast Metabolism" (Mills, A.K. Ed) 306 (Blackwells Scientific Publications, Oxford).

Levin, R. (1958). In "The Pharmacy of Silicones" (The Chemist and Druggist, London) 35.

Lewis, C.W. (1974). Ph.D. Thesis, University of Strathclyde, Glasgow.

Lindegren , C.C. & Lindegren, G. (1943). Proc. Nat. Acad. Sci. (US) *29*, 306.

Lindquist, W. (1953). J. Inst. Brew. *59*, 59.

Lindstedt, G. (1945). Arkir, Kemi. Mineral Geol. *20* A, 1.

Lowry, O.H., Roberts, N.R., Leiner, K.Y., Wu, M.L. & Marr, A.L. (1954). J. Biol. Chem. *207*, 1.

Lüers, H. & Geys, K. (1922). Kolloidzschr *30* , 372.

Lyons, T.P. & Hough, J.S. (1970a) Biochem. J. *119*(3), 10p.

Lyons, T.P. & Hough, J.S. (1970b). J. Inst. Brew. *76* , 564.

Lyons, T. P. & Hough, J.S. (1970c). Brewer's Digest *45*(8), 52.

Lyons, T.P. & Hough, J.S. (1971). J. Inst. Brew.*77*, 300.

MacWilliam, I.C. (1970). J. Inst. Brew. *76*(6), 524.

Manners, D.J. & Masson, A.J. (1969). FEBS Letters *4*, 122.

Manners, D.J., Masson, A.J. & Patterson, J.C. (1973a). Biochem. J. *135*, , 19.

Manners, D.J., Masson, A.J., Patterson, J.C., Björndal, H. & Lindberg, B. (1973b). Biochem. J. *135*, 31.

Manners, D.J. & Patterson, J.C. (1966). Biochem. J. *98*, 19c.

Marshall, H.S.B. & WIlliams, T.R. (1966). Br. Pat. 1,020,022.

Masschelein, C.A. (1959). Rev. Ferment. Ind. Ailiment. *14*, 59, 87.

Masschelein, C.A., Jeunehomme-Ramos, C.,Castiau, C. & Devraux, A. (1963). J. Inst. Brew. *69*, 332.

McMurrough, I. & Rose, A.H. (1967). Biochem. J. *105*, 189.

Mel'tser, I.A. & Kuramshin, Yu. N. (1958). Khlebopekar i Konditer Prom. *2* (10), 13.

Mill, P.J. (1964a). J. Gen. Microbiol. *35*, 53.

Mill, P.J. (1964b). J. Gen. Microbiol. *35*, 61.

Mill, P.J. (1966). J. Gen. Microbiol. *44*, 329.

Miller, M.W. & Phaff, H.J. (1958). Antonie van Leewenhoek *24*, 225.

Misaki, A., Johnson, Jr, J. Kirkwood, S., Scaletti, J.V. & Smith, F. (1968). Carb. Res. *6*, 150.

Misaki, A. & Smith, F. (1963). Abstr. Amer. Chem. Soc. 144th meet. 14c.

Morel, P. (1958). Chem. Abstr. *52*, 758.

Morris, E.O. (1966). In "The Fungi" Vol. 2 (The Fungal Organisms; Ainsworth, G.C. & Sussman, A.S. eds.) 63 (Academic Press, New York.)

Mundkur, B. (1960). Expl. Cell. Res. *20*, 28.

Nagasaki, S., Neumann, N.P., Arnow, P., Schnable, L.D. & Lampen, J.O. (1966). Biochem. Biophys. Res. Commun. *25*, 158.

Nakajima, T. & Ballou, C.E. (1974a). J. Biol. Chem. *249*, 7679.

Nakajima, T. & Ballou, C.E. (1974b). J. Biol. Chem. *249*, 7685

Neal, P. (1969). J. Ass. Offic. Anal. Chem. *52* (4), 875.

Neumann, N.P. & Lampen, J.O. (1967). Biochemistry NY *6*, 468.

Nickerson, W.J. (1963). B acteriol. Rev. *27*, 305.

Nickerson, W.J. (1964). In "Cellular Membranes in Development" (Locke,M.ed). 281. (Academic Press, New York).

Nickerson, W.J. & Falcone, G. (1956a). Science NY *124*, 318.

Nickerson, W.J. & Falcone, G. (1956b). Science NY *124*, 722.

Nielsen, N. (1937). Compt. Rend. Trav. Lab. Carlsberg. Ser. Physiol. *22* (4), 61.

Noll, W. (1968). In "Chemistry and Technology of Silicones", 246, (Academic Press, London).

Northcote, D.H. (1963). Pure Appl. Chem. *7*, 669.

Northcote, D.H. & Horne, R.W. (1952). Biochem. J. *51*, 232.

Patching, J.W. & Rose, A.H. (1970). In "Methods in Microbiology" (Norris, J.R. & Ribbons, D.W. eds) Vol. 2, 23 (Academic Press, London).

Patnode, W.I. & Wilcock, D.F. (1946). J. Amer. Chem. Soc. *68*, 358, 360.

Pauling, L. (1947). In "General Chemistry" (Freeman, U.S.A) 869.

Peat, S., Whelan, W.J. & Edwards, T.E. (1958a). J. Chem. Soc. 3862.

Peat, S., Turvey, J.R. & Evans, J.M. (1958b). J. Chem. Soc. 3868.

Peat, S., Whelan, W.J. & Edwards, T.E. (1961a). J. Chem. Soc. 29.

Peat, S., Turvey, J.R. & Doyle, D. (1961b). J. Chem. Soc. 3918.

Pfeiffer, H. (1932). Protoplasm. *15*, 590.

Phaff, H.J. (1963). A. Rev. Microbiol. *17*, 15.

Phaff, H.J. (1971). In "The Yeasts" Vol. 2 (Rose, A.H. & Harrison, J.S. eds) 135 (Academic Press).

Preiss, J.W. (1958). Arch. Biochem. Biophys. *75*,186.

Rainbow, C. (1966). Proc. Biochem. *1* , 489.

Rainbow, C. (1970). In "The Yeasts" Vol. 3 (Rose, A.H. & Harrison, J.S. eds) 147 (Academic Press).

Raschke, W.C . & Ballou, C.E. (1971). Biochemistry *10* , 4130.

Raschke, W.C., Kern, K.A., Antalis, C. & Ballou, C.E. (1973). J. Biol. Chem. *248*, 4660.

Richards, M. & Cowland, T.W. (1967). J. Inst. Brew. *73*, 552.

Roelofsen, P.A. (1953). Biochim. Biophys. Acta. *10*, 477.

Rose, A.H. (1963). Wallerstein Labs. COmmun. *26*, 21.

Rosenfeld, L. (1974). PhD. Thesis, University of California, Berkeley.

Rosenfeld, L. & Ballou, C.E. (1974). J. Biol. Chem.*249*,2319.

Ross, S. (1967). Chem. Engng. Progr.*63*,9.

Rothstein, A. (1955). In "Electrolytes in Biological System" (Shanes, A.M. ed) 65 (American Physiological Society).

Rothstein, A. & Hayes, A.D. (1956). Archs. Biochem. Biophys. *63*, 87.

Rowe, V.K. (1948). J. Ind. Hyg. and Toxicol. , 332.

Ruiz Hernandez, M. (1964). Semana Vitivinicola No. 922, 4.

Ruiz Hernandez, M. (1968). Semana Vitivinicola 23 (1.139) 1.937 1.939, 1.941.

Ruiz Hernandez, M. (1970). Semana Vitivinicola 25 (1.256), 3.141, 3.43.

Salkowski, E. (1894a) Ber. dt. Chem. Ges. *27*, 3325.

Salkowski, E. (1894b). Ber. dt. Chem. Ges. *27*, 497, 925.

Sauer, R.O. & Mead, D.J. (1946). J. Amer. Chem. Soc. *68* , 1794.

Schwarz, E.G. & Ried, W.G. (1964). Ind. Engng. Chem. *56*, 9.

Searcy, R.L., Reardon, J.E. & Foreman, J.A. (1967). Amer. J. Medic. Tech. *33*, 15.

Sentandreu, R. & Northcote, D.H. (1968). Biochem. J. *109* , 419.

Sentandreu, R. & Northcote, D.H. (1969). Carb. Res. *10*, 584.

Shaw, D.J. (1970). In "Instrocution to Colloid and Surface Chemistry" 206 (Butterworths, 2nd edition).

Shaw, N. & Stead, A. (1974). B iochem. J. *143*, 461.

Siebert, F.B. & Atno, J. (1946). J. Biol. Chem. *163*, 511.

So, L.L. & Godlstein, I.J. (1968). J. Biol. Chem. *243*, 2003.

Soifer, R.D. (1966). Med. Prom. SSSR *3*, 22.

Somers, E. & Fisher, D.J. (1967). J. Gen. Microbiol. *48*, 147.

Sorenson, S. (1909). Biochem. Z. *21*, 131.

Sorenson, S. (1912). Ergebn. Physiol. *12*, 393.

Stewart, G.G. (1972). Tech. Quart. Master Brew. Assn. Amer. *9*(2), xxv.

Stewart, G.G. (1975). The Brewers Digest (March) 42.

Stewart, T.S. & Ballou, C.E. (1968). Biochemistry NY *7*, 1855.

Stewart, T.S., Mendershausen, P.B. & Ballou, C.E. (1968). Biochemistry NY *7*, 1843.

Stewart, G.G. & Garrison, I.F. (1972). Proc. Amer. Soc. Brew. Chem. 118.

Stewart, G.G., Russell, I. & Garrison, I.F. (1973). Proc. Amer. Soc. Brew. Chem. 100.

Stewart, G.G., Russell, I. & Garrison, I.F. (1975). J. Inst. Brew. *81*, 248.

St Johnston, J.H. (1953). Wallerstein Labs. Commun. *16*, 39.

Stockhausen, F. (1927). Wochensch. Brau. *44*, 121, 133.

Suomalainen, H., Nurminen, T. & Oura, E. (1967). Suomen. Kemi. B*40* , 323.

Sutton, D.D. & Lampen, J.O. (1962). Biochim. Biophys. Acta. *56*, 303.

Suzuki, S. & Sunayama, H. (1968). Jap. J. Microbiol.*12* , 413.

Suzuki, S., Sunayama, H. & Saito, T. (1968). Jap. J. Microbiol. *12* , 19.

Tanaka, H. (1963). PhD. Thesis, University of California, Davis.

Tarentino, A.L., Plummer, T.H. & Maley, F. (1974). J. Biol. Chem. *249*, 818.

Taylor, N.W. & Orton, W.L. (1973). J. Inst. Brew. *79*, 294.

Taylor, N.W. & Orton, W.L. (1975). J. Inst. Brew. *81*, 53.

Thieme, T.R. & Ballou, C.E. (1971). Biochemistry. *10* , 4121.

Thorne, R.S.W. (1951a). Compt. Rend. Lab. Carlsberg. Ser. Physiol. *25*, 101.

Thorne, R.S.W. (1951b). Proc. Euro. Brew. Conv., Brighton, 21.

Thorne, F.R.W. (1952). Wallerstein Labs. Commun.*15*, 201.

Thorne, R.S.W. (1972). Tech. Quart. Master Brew. Assn. Amer. *9*, 173.

Tkacz, J.S., Cybulska, E.B. & Lampen, J.O. (1971). J. Bacteriol. *105*, 1.

Vernon, P.S. & Rose, A.H. (1976). In preparation (to be published in J. Inst. Brew).

Wickerham, L.J. (1951). U.S. Dept. Agr. Tech. Bull. No. 1029.

Wiles, A.E. (1951). Proc. Euro. Brew. Conv., Brighton, 84.

Williams, N.J. & Wiseman, A. (1973). Fed. Euro. Biochem. Soc. Abstr. Comm., Session 14.

Windisch, S. (1968). Brewers Digest *43*(11), 62.

Winge, O. & Lausten, O. (1937). Compt. Rend. Lab. Carlsberg *22*(6), 99.

Winge, O. & Lausten, O. (1938). Compt. Rend. Lab. Carlsberg *22*(6), 235.

Wood, W.A. (1966). In "Methods in Enzymology" (Colowick, S.P. & Kaplan, H.O. eds). Vol. IX, 743 (Academic Press, New York).

Woof, J.B. (1962). J. Inst. Brew. *68*, 315.

Zechmeister, L. & Toth, G. (1934). Biochem. Z. *270*, 309.

Zechmeister, L. & Toth, G. (1936). Biochem. Z. *284*, 133.

Zalay, L. Gebhardt, I., Kiss, I., Vaghy, T. & Inezefi, I. (1965). Zentr. Bakteriol. Parasitenk. Abt. Ong., 197(1).